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E. Coli Antibodies Do Not Cause False-positivity in Recombinant Anti-HIV Assays

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129 sera with known antibody titres against E. coli 026 and E. coli 055 strains were tested with the Abbot second generation anti-HTLV III recombinant screening assay. No difference in the O.D. values was found between sera with high, normal and low anti-E. coli titres. In addition, no false-positive reactions were observed with the anti-HIV negative sera containing E. coli antibodies in high titres in a Western blot assay in which recombinant env antigen was applied. These results suggest that E. coli assays in which E. coli-produced recombinant antigens are used.

Keywords: E. coli antibodies, recombinant anti-HIV assay, false positivity

Introduction

As a result of the development of HIV serology, improved, second generations ELISA tests are more and more frequently used for screening blood donors and members of high risk groups for HIV antibodies. In most second generation assays, plates or beads are coated with recombinant env and/or core antigens. In addition, recombinant HIV antigens are applied in some experimental Western blot assays as well [2, 5]. Use of these antigens excludes the possibility of false-positive reactions due to anti-HLA and other anti-lymphocyte antibodies [3, 4, 6]. Since, however, recombinant antigens are produced mostly in different strains of E. coli, theoretically it is possible that some antigens of bacterial origin can be present even in the purified HIV env and core antigen preparations. If recombinant HIV antigens are really contaminated with bacterial antigens, false-positive anti-HIV reactions can develop when serum samples containing anti-E. coli antibodies in high titres are tested.

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In order to address this possibility, we have investigated 129 sera with known titres against two E. coli strains in the recombinant anti-HTLV III screening assay of Abbott, strictly according to the recommendations of the manufacturer. A part of these sera were tested in a recombinant HIV env Western blot assay (PEX-41) as well. No false-positive reactions were obtained in either type of anti-HIV assays.

Materials and Methods

Serum samples tested

The sera used were obtained from 129 patients with different rheumatic diseases, mostly with juvenile and adult rheumatoid arthritis.

Measurement of E. coli antibodies

Antibodies against E. coli 026 and E. coli 055 strains were measured by passive haemagglutination [1] and were expressed as $\log_2$ of the highest serum dilution showing a haemagglutination reaction. Normal values for these antibacterial antibodies were determined in the sera of 22 blood donors.

ELISA assay with recombinant antigen

Serum samples were tested in the Abbott recombinant HTLV III EIA kit strictly according to the recommendations of manufacturer.

PEX-41 Western blot assay

The recombinant HIV env protein PEX-41 expressed in E. coli POP 2136 [5] was separated in SDS-PAGE using a discontinous system, with a 4.5% starting gel and a 12.5% separating gel. The sample was electrophoresed for 5 hours at 35 mA. The proteins were transferred electrophoretically to nitrocellulose paper in a Bio-Rad Transblot apparatus overnight at 250 mA. Before using the strips, they were incubated in PBS containing 5% skim milk and 0.1% Triton X-100 for 1 hour to block aspecific binding sites. Next, the strips were incubated in the tested sera, washed, incubated with horseradish peroxidase conjugated anti-human IgG, washed 3 times and stained with diaminobenzidine tetrahydrochloride. The washing procedure was done in the above mentioned buffer except the last wash which was in PBS.

Results

Titres of E. coli antibodies in the sera of blood donors

Titres of E. coli antibodies expressed in $\log_2$ dilution values were determined in sera of 22 blood donors. Normal values (mean $\pm$ 2 S.D.) were found to be 4.13 $\pm$ 1.5 for the E. coli 026 and 3.45 $\pm$ 1.94 for the E. coli 055 antibodies. On the basis of these data, anti-026 titres of $\leq$ 2.5, 3 to 5.5, and $\geq$ 6.0 were considered as low, normal, and high, respectively. For the anti-055 titres, the same categories were defined as $\leq$ 1.5, 2 to 5, and $\geq$ 5.5, respectively.

Comparison of serum samples containing E. coli antibodies in low, normal and high titres in a recombinant HIV screening assay

No O.D. value above cut-off O.D. was observed in any of the sera tested. No significant differences were observed in the O.D. values when sera with low, normal, and high level of anti-E. coli antibodies were compared to each other (Table 1). Similarly, there was no significant negative or positive correlation between O.D. values in the Abbott assay and the titres of either the E. coli 026 or E. coli 055 antibodies (correlation coefficients were –0.05, and 0.13, respectively), whereas a significant correlation (R = 0.45) was observed between anti--026 and anti-055 titres.

Table 1

O. D. values obtained in the Abbott recombinant anti-HIV screening assay with sera containing antibodies against two E. coli strains in different amounts

Level of E. coli antibodies	Anti-026		Anti-055	
	number of sera	O. D. value mean $\pm$ S. D.	number of sera	O. D. value mean $\pm$ S. D.
Low*	16	0.18 $\pm$ 0.06	21	0.14 $\pm$ 0.06
Normal**	76	0.13 $\pm$ 0.07	80	0.14 $\pm$ 0.08
High***	37	0.15 $\pm$ 0.06	28	0.15 $\pm$ 0.04

* $\leq$2,5; $\leq$1.5, ** 3 – 5.5; 2 – 5, *** $\geq$6.0; $\geq$5.5 for the anti-026 and anti-055 antibodies, respectively

Study of sera with low and high anti-E. coli titres in a recombinant Western blot assay

Nine and 8 sera, respectively with low and high anti-026 and/or anti-055 titres were selected for investigation in the PEX-41 Western blot assay (Fig. 1). On the control strip incubated with a confirmed anti-HIV positive serum sample,

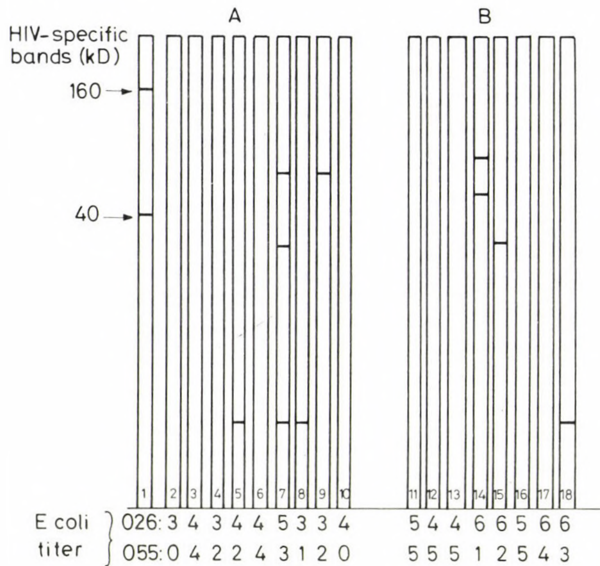

Fig. 1. Western blot reaction performed with recombinant-derived HIV envelope antigens. The strips were incubated with a confirmed HIV-seropositive serum sample (first strip to the left), with HIV-seronegative serum samples containing E. coli 026 and/or 055 antibodies in low titres (9 samples, A) or high titres (8 samples, B)

2 bands could be seen with an apparent molecular weight (m. w.) of 160, and 40 kD. No bands of the same m. w. were observed on the strips incubated with the 17 serum samples with known anti-E. coli levels. Several aspecific bands were observed on these strips; there was no difference in the number or intensity of these bands when the groups of sera with low and high titres were compared, although great variations were found among the individual sera in both groups.

Discussion

According to the present work, antibodies against two E. coli strains, E. coli 026 and E. coli 055 do not induce false-positive reactions in a recombinant anti-HIV enzyme immunoassay. Since antibodies against different E. coli strains cross-react with each other (a strong correlation between anti-026 and 055 antibodies was found in this study as well) our results suggest that E. coli antibodies present in the tested sera do not disturb HIV-serology performed with solid phase enzyme immunoassays using E. coli-produced recombinant antigens.

In the PEX-41 recombinant Western blot assay, no sera, not even those containing E. coli antibodies in high titre, showed the two bands (160 and 40 kD) characteristic for the assay [2, 5]. (The 160 kD protein consists of the HIV env

fragment and the 117 kD cro-beta-gal fusion protein derived from the vector PEX-2, whereas the 40 kD protein is thought to correspond to the isolated env fragment [5].) The aspecific bands observed on the strips incubated with the HIV negative sera probably represent E. coli antigens, since we have observed similar pattern in Western blot test performed with an other E. coli-expressed HIV--antigen preparation, too. Since, however, the aspecific bands are not in the same m. w. range as the specific 160 and 40 kD bands, their presence did not disturb evaluation of the PEX-41 Western blot assay. False-positivity may have serious consequences at anti-HIV screening and confirmation in the low-risk populations [6], therefore our present findings may have practical importance.

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Haematologia 23 (1), pp. 9—19 (1990)

1,25-Dihydroxyvitamin D_3 Induces Normal Mononuclear Blood Cells To Differentiate in the Direction of Monocyte-Macrophages

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(Received 17 August, 1988; accepted 13 January, 1989)

Normal blood mononuclear cells incubated in the presence of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] in suspension culture showed increased differentiation to adherent macrophages. The effect was specific to 1,25-$(OH)_2D_3$ as the closely related metabolite 25-hydroxyvitamin D_3 did not increase macrophage numbers. Giant multinuclear cells were observed in increased numbers in 1,25-$(OH)_2D_3$-treated cultures. However this effect was not specific to 1,25-$(OH)_2D_3$ but due to cell crowding as these cells were also plentiful when cultures without 1,25-$(OH)_2D_3$ were incubated at high cell densities. Increased numbers of macrophages were seen after six days of incubation; this effect of 1,25-$(OH)_2D_3$ occured also if the mononuclear cells were depleted of T-cells or exposed to X-irradiation at doses up to 25Gy. Addition of 1,25-$(OH)_2D_3$ to cultures in semi-solid media did not, however, increase the numbers of monocyte-macrophage colonies. These results suggest that the target cell for 1,25-$(OH)_2D_3$ is of the monocyte-macrophage lineage and is at a relatively late stage of maturation.

Keywords: monocyte-macrophage differentiation, 1,25-dihydroxyvitamin D_3.

Introduction

1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) the active hormonal metabolite of vitamin D_3 has multiple effects on hematopoiesis in vitro. It induces human promyelocytic leukemia (HL-60) cells to differentiate to monocytes and macrophages [1, 2]; this event is associated with decreased expression of the proto--oncogene c-myc [3]. It also suppresses in vitro proliferation of megakaryocytes [4] and fibroblasts [5] from normal human bone marrow. 1,25-$(OH)_2D_3$ induces normal bone marrow cells to differentiate in the direction of monocytes and macrophages in vitro [1, 6] and we here report that 1,25-$(OH)_2D_3$ has a similar but not identical effect on mononuclear cells taken from normal human peripheral blood. We do not know precisely what type of cell differentiates in response to

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1,25-$(OH)_2D_3$. Previous work has suggested that relatively late precursors and even cells such as promyelocytes and myelocytes, conventionally considered to have undergone committment to the granulocytic pathway of differentiation, may be induced by 1,25-$(OH)_2D_3$ to differentiate in the direction of monocytes and macrophages [7]. Monocytes and activated T- and B-lymphocytes have receptors for 1,25-$(OH)_2D_3$ [8] but the absence of receptor for 1,25-$(OH)_2D_3$ does not preclude the possibility of an effect: a non-receptor mediated mechanism for monocytic differentiation of HL-60 cells has been proposed [9]. We have attempted to identify the target cell by observing the effect of 1,25-$(OH)_2D_3$ on initially adherent, non-adherent and T-cell depleted mononuclear cells. Also, by observing the period of time taken for maximal numbers of monocytes to form we have attempted to estimate how far the target cell is removed along the differentiation pathway from the mature monocyte.

Materials and Methods

Isolation of cells

Blood was taken from normal volunteers into preservative-free heparin (Paines and Byrne Ltd, Greenford, Middx, UK) 10 iu/ml. The blood was layered onto lymphoprep (Nyegaard Ltd, Oslo, Norway) and centrifuged at 800*g* for 20 minutes. The mononuclear cell layer was then washed and resuspended at a cell concentration of 1×10^9/l in McCoys 5A medium (Flow Labs, UK) supplemented with 15% pooled, heat-inactivated human serum.

Incubation method

1,25-$(OH)_2D_3$ (Roche Products Ltd, UK) was initially dissolved in ethanol then further diluted to suitable concentrations in culture medium. 50–100 μl of solution were added to 10 ml cultures of mononuclear blood cells. Control cultures were incubated with the same volume of 25-hydroxyvitamin D_3 (25-OHD_3) or ethanol.

The cultures were incubated in 25 cm^3 tissue culture flasks in a 5% CO_2/air atmosphere at 37 °C for variable intervals. For some experiments the non-adherent cells were collected after 24 hours and incubated separately. The remaining adherent cells were washed once in situ and fresh McCoys medium containing 15% human serum (10 ml) was added. Incubation was continued as above with 1,25-$(OH)_2D_3$ or 25-OHD_3.

Irradiation

The culture flasks to be irradiated were exposed to X-irradiation from a Caesium-137 source in a Gammacell-1000 blood irradiator (Atomic Energy

of Canada Ltd, Ottawa, Canada) for a suitable period of time (0–6 mins) at the start of the incubation period.

Assessment of monocyte-macrophage (MM) numbers

When the incubation period was complete non-adherent cells were decanted and the adherent cell layer was washed with McCoys 5A medium. The cells were stained with May-Grunwald Giemsa and in some cases with Sudan Black and for α-naphthyl-acetate esterase (ANAE) by standard techniques [10]. An area 7 cm × 5 cm was marked on the outside of the flask and all the cells within that area were counted by light microscopy. The number of adherent cells was expressed as cells × $10^5/m^2$.

Granulocyte-macrophage colony assay

The technique of Pike and Robinson [11] was used with minor modifications. Blood was obtained from normal laboratory volunteers and mononuclear cells separated over Lymphoprep (Nyegaard, Oslo). Peripheral blood mononuclear cells (0.5×10^6/ml) were incubated with giant-cell conditioned medium (GCT-CM; Gibco Ltd, UK; 20% v/v) in 35 mm^2 culture dishes (Sterilin, UK). Pooled human serum was used throughout. After 10 days incubation at 37 °C and 5% CO_2 the agar gel was transferred to a glass slide, dried and stained with May-Grunwald Giemsa and for α-naphthyl-acetate esterase activity. Results are expressed as mean and standard deviations of triplicate cultures from 6 donors for total groups (> 10 cells). Colonies were not specifically counted as their numbers were so low.

T-cell depletion

T-cells were depleted by rosetting with sheep red blood cells (SRBC: Tissue Culture Services, Slough, UK). Briefly, mononuclear cells (15 × 10^9/l) were centrifuged with AET-treated SRBC (10% SRBC in 40% fetal calf serum/phosphate-buffered saline) at 100*g* for 5 minutes. The button was resuspended, the suspension layered on lymphoprep and centrifuged at 600*g* for 20 minutes. The mononuclear layer was washed twice with Hanks BSS. The mononuclear cells were assessed for residual T-cells by using an Immune Monotoring Kit in a FACS-analyser I (Becton-Dickenson, Mountain View, California 94039).

Results

Monocyte-macrophage numbers

The number of MM adherent cells after 14 days in culture was highly variable between individuals but in all cases the number was greatly increased by addition of 1,25-$(OH)_2D_3$. There was an approximate dose-response relationship

Table 1

Monocyte-macrophage numbers in cultures of peripheral mononuclear cells incubated vitamin D_3 metabolites for 14 days
Number of monocyte-macrophages*

Experiment number	Incubation Conditions			
	Level of 1,25-$(OH)_2$ D_3(M)	Control	25-OHD_3	1, 25-$(OH)_2D_3$-treated
1	10^{-7}	280*	310	1345
	10^{-8}	510	605	1100
	10^{-9}	425	450	1050
2	10^{-7}	30	61	680
	10^{-8}	45	40	350
	10^{-9}	40	35	275
3	10^{-7}	320	360	1050
	10^{-8}	380	500	1180
	10^{-9}	400	410	995

% increase in macrophage numbers with 1, 25-$(OH)_2D_3$**
10^{-7}M — 1022 ± 627
10^{-8}M — 435 ± 174
10^{-9}M — 311 ± 63

* Each figure represents the numbers of monocyte-macrophages which were adherent in the flask expressed as cells$\times 10^5/m^2$.

** Each figure represents the percentage increase in the number of monocyte-macrophages (mean $\pm$ SEM) compared to control numbers. These increases are significant with $P < 0.025$ (10^{-7}, 10^{-8}) and $P < 0.05$ (10^{-9}). Control cultures contained the same quantity of ethanol vehicle as did the cultures which contained 25-OHD_3 or 1, 25-$(OH)_2D_3$.

in the range of 10^{-9}–10^{-7} M. MM numbers were the same in cultures containing ethanol or 25-OHD_3 (Table 1). In both control and treated cultures MM numbers were maximal at day 14 although the maximum difference in numbers between control and treated cultures occurred at day 20. At all of the time points studied from days 6 to 20 MM numbers were significantly higher in the 1,25-$(OH)_2D_3$-treated cultures ($P < 0.01$, $n = 5$) (Fig. 1). Because of the wide variation in MM numbers between individuals the data were processed using the paired Student *t*-test. There was no difference detectable at days 1 and 3. The ANAE reaction was strongly positive in more than 80% of MM.

Monocyte-macrophage numbers after irradiation

X-irradiation of the mononuclear blood cells before incubation decreased the number of MM in both control and 1,25-$(OH)_2D_3$-treated cultures (Fig. 2). However after irradiation to 25Gy the 1,25-$(OH)_2D_3$-treated cultures contained

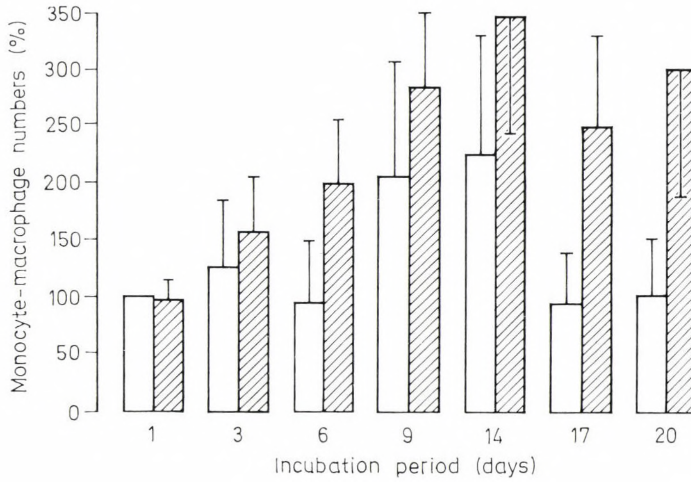

Fig. 1. MM numbers from days 3 to 20 (mean $\pm$ SD, n = 5). Results are expressed as a percentage of the control figure on day one. Clear bars represent control cultures containing 25-OHD_3 and hatched bars represent cultures containing 1,25-$(OH)_2D_3$ (10^{-8} M)

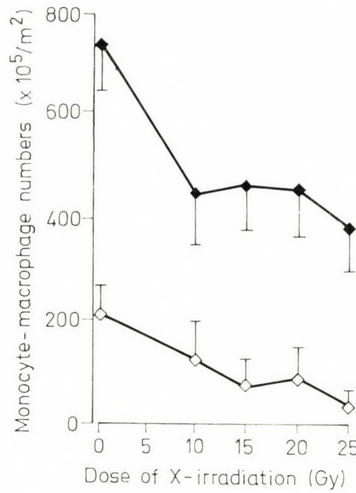

Fig. 2. MM numbers after exposure of cultures at the start of incubation to X-irradiation (mean $\pm$ SEM, n = 3). Black diamond sign indicates cultures incubated with 1,25-$(OH)_2D_3$ (10^{-8} M). White diamond sign indicates cultures incubated with 25-OHD_3. The incubation period was 14 days

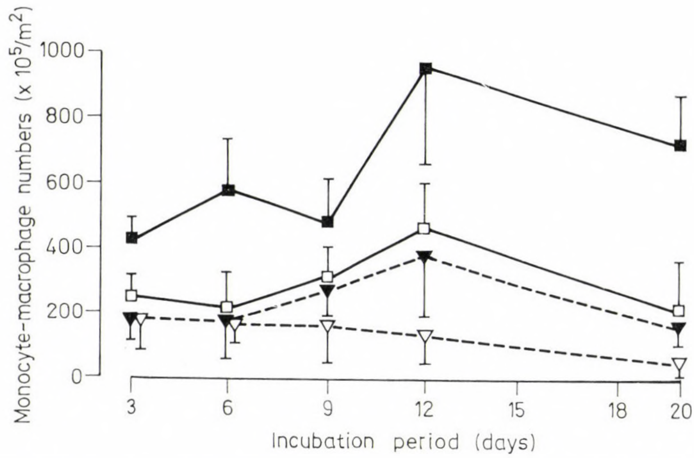

Fig. 3. MM numbers after various incubation periods. Solid lines indicate unirradiated cultures. Broken lines indicate cultures exposed to 25Gy X-irradiation before the start of incubation. Black square or triangle indicate cultures with $1,25\text{-}(OH)_2D_3$ (10^{-8} M). White square or triangle indicate cultures with $25\text{-}OHD_3$ (10^{-8} M). Results represent mean ± SEM, n = 4

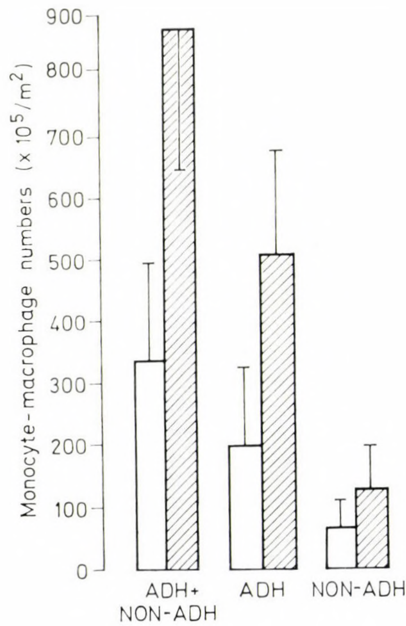

Fig. 4. MM numbers after 14 days in cultures of adherent, non-adherent and mixed adherent and non-adherent cells. Results represent mean ± SEM, n = 3. Clear bars represent control cultures containing $25\text{-}OHD_3$ (10^{-8} M) and hatched bars represent cultures containing $1,25\text{-}(OH)_2D_3$ (10^{-8} M)

approximately 10 times as many MM as controls when assessed after 12 days incubation. In fact even after 50Gy MM numbers were still increased after incubation with 1,25-$(OH)_2D_3$ in comparison to the control numbers (results not shown). Some multinucleate giant cells were seen in the irradiated cultures especially in areas where the cells were crowded.

MM numbers were increased to a significant extent by 1,25-$(OH)_2D_3$ after only six days incubation, but X-irradiation (25Gy) prevented this initial increase. Cultures examined after longer periods of incubation clearly contained more MM in the presence of 1,25-$(OH)_2D_3$ than the controls despite irradiation ($P < 0.05$, $n = 4$ for days 12, 20) (Fig. 3).

Multinucleate giant cells

Multinucleated giant cells were increased in number in cultures which contained 1,25-$(OH)_2D_3$ but when the mononuclear cells at very low density (starting concentration 5×10^7/l) were incubated with 1,25-$(OH)_2D_3$ a very much lower proportion of multinucleate cells were seen mainly in crowded areas of the flasks. Irradiation to 25Gy did not prevent their formation.

Monocyte-macrophage numbers after adherence and T-cell depletion

When initially adherent cells were co-cultured with initially non-adherent cells the expected rise in macrophage numbers with 1,25-$(OH)_2D_3$ occurred. A similar but lesser rise occurred when non-adherent cells alone were cultured with 1,25-$(OH)_2D_3$ ($P < 0.05$, $n = 3$). The small 1,25-$(OH)_2D_3$-induced rise seen with initially non-adherent cells was not statistically significant (Fig. 4).

The number of monocytes in the non-adherent fraction was 2.6 ± 0.9 ($\times 10^7$/l) on day 1, 3.2 ± 0.8 ($\times 10^7$/l) on day 20 in control cultures and 7.3 ± 1.8 ($\times 10^7$/l) on day 20 in cultures treated with 1,25-$(OH)_2D_3$. (Control v treated

Table 2

Effect of T-cell depletion on monocyte-macrophare numbers

	Untreated	T-cell depleted
Control*	100**	95 ± 8
1,25-$(OH)_2D_3$	129 ± 5	122 ± 6

* Control cultures contained equal quantity of ethanol vehicle as those containing 1,25-$(OH)_2D_3$.

** Figures represent MM numbers expressed as percentage (mean $\pm$ SEM, $n = 3$) of untreated control figures.

Table 3

Types of colony grown from peripheral blood mononuclear cells (numbers/2×10^5 cells; M $\pm$ SD, n = 6)

	Monocytic	Granulo-cyte-macrophage	Neutrophil	Eosinophil
Control*	3 ± 2	2 ± 2	5 ± 3	10 ± 5
1,25-$(OH)_2D_3$(10^{-8}M)	4 ± 1	2 ± 2	6 ± 4	9 ± 4

* Ethanol vehicle (0.1% v/v) was added.

$P < 0.01$, n = 3). Depleting the cultures of T-cells did not prevent the increase in MM numbers with 1,25-$(OH)_2D_3$ (Table 2) despite the fact that 98% of the T-cells were removed.

CFU–GM assay (Table 3)

The 1,25-$(OH)_2D_3$ treated cultures contained 4 ± 1 (n = 6) monocyte--macrophage clusters and colonies compared to 3 ± 2 in the control (ethanol vehicle alone) cultures. The majority of non-MM colonies were eosinophilic and the corresponding numbers were 17 ± 4 (1,25-$(OH)_2D_3$) and 17 ± 4 (control).

Discussion

The increase in MM numbers which we observed in the cultures incubated with 1,25-$(OH)_2D_3$ was clearly a specific response because the closely related vitamin D_3 metabolite 25-OHD_3 did not cause any increase. This strongly suggests that the effect is mediated by binding of 1,25-$(OH)_2D_3$ to a specific receptor in the responsive cell. Monocytes and probably their immediate precursors contain 1,25-$(OH)_2D_3$-receptors and cells of this lineage are therefore most likely to be the immediately responsive cells. This interpretation is supported by our findings that the MM numbers induced from initially adherent cells were very much greater than those from initially non-adherent cells and not much less than the numbers induced by 1,25-$(OH)_2D_3$ from the total mononuclear cell population. 1,25-$(OH)_2D_3$ is not simply an adhesion-inducing agent because the numbers of MM in the non-adherent fraction were also increased. Although activated T-lymphocytes contain receptors for 1,25-$(OH)_2D_3$ and conceivably could produce a lymphokine in response to 1,25-$(OH)_2D_3$ that induced precursor cells to differentiate to MM our finding that depleting the mononuclear cells of T-cells had no detectable impact on the inductive effect of 1,25-$(OH)_2D_3$ is not readily compatible with this idea.

The cell which is stimulated to differentiate by 1,25-$(OH)_2D_3$ is likely to be a relatively late MM-precursor which does not require to undergo mitosis before it becomes identifiable as an adherent monocyte-macrophage. This is so firstly because the numbers of MM were clearly increased as early as six days after adding 1,25-$(OH)_2D_3$, secondly because the numbers of MM colonies were not increased by 1,25-$(OH)_2D_3$ and thirdly because a substantial proportion of MM derived from the stimulated cells even after irradiation. Although irradiation to 25Gy (and in some experiments to 50Gy) reduced the numbers of MM formed in response to 1,25-$(OH)_2D_3$ these numbers were still increased in comparison to the control cultures. Irradiation to much less than this level completely ablates formation of haemopoietic colonies in vitro. Whether the inducing effect is entirely to influence cells to mature to adherent MM is not clear. Although the well documented ability of 1,25-$(OH)_2D_3$ to induce maturation and adherence of other cell types such as HL-60 cells may suggest that this is also its primary role in the present system it remains possible that additional mechanisms such as continuing activation of the MM are involved.

There was no detectable increase in the size or number of the cell groups in 1,25-$(OH)_2D_3$-treated cultures. In contrast MM colony formation from normal human bone marrow MNC is induced by 1,25-$(OH)_2D_3$ [4, 6]. This apparent difference may be explained by the fact that MM-colony-forming cells exist in numbers which are too low in peripheral blood for an effect to be detectable. However marrow and blood progenitor cells differ in many respects [12, 13, 14], and varying responses to differentiation inducers may result from differing age distributions. In a murine system Nagler et al. [11] also recently found that whereas low doses of cytosine arabinoside increased the numbers of MM in liquid cultures they did not alter the numbers of MM colonies.

The number of multinucleate giant cells [15] in related culture systems is increased by 1,25-$(OH)_2D_3$, a finding which lends support to the disputed idea that these cells are closely related to osteoclasts. Although we observed an increase in the multinucleate cells in cultures with 1,25-$(OH)_2D_3$ this effect appears to result from crowding of macrophages as a result of their increased number rather than from any specific reaction of 1,25-$(OH)_2D_3$ because the proportion of multinucleate cells was much less when cells were cultured at a lower concentration. Our data support the notion that cell fusion is the mechanism responsible for multinucleation in culture because irradiation which should prevent endomitosis did not prevent formation of multinucleate giant cells.

These results are similar to those reported for normal human bone marrow cells and for peripheral blood cells from patients with chronic granulocytic leukaemia. In those studies we identified the cells positively as MM by functional and cytochemical criteria and by identifying surface antigens specific for MM with a panel of monoclonal antibodies. In this study the cells were indistinguishable by morphological and cytochemical criteria from those we have previously reported.

It is not clear if this in vitro effect of 1,25-(OH)$_2$D$_3$ has a counterpart in vivo. On the one hand we used concentrations of 1,25-(OH)$_2$D$_3$ one to two logs higher than circulating levels. On the other hand the number of MM is a crude measure of effect, and the dissociation constant (Kd) for 1,25-(OH)$_2$D$_3$ in monocytes [8] and in CGL buffy coat cells [16] is close to the physiological range although a higher Kd was found for HL-60 cells [17]. Also monocytes may synthesize 1,25-(OH)$_2$D$_3$ and in this synthesis may powerfully be stimulated by γ-interferon [18]. Thus concentrations of 1,25-(OH)$_2$D$_3$ in the microenvironment of developing monocytes in vivo could lie within the concentration range employed in our experiments. Low circulatory levels have been reported in leukaemia [19] and responses to treatment with 1,25-(OH)$_2$D$_3$ have been described in myelofibrosis with rickets [20, 21, 22]. However whether 1,25-(OH)$_2$D$_3$ has a physiological role as a modulator of haematopiesis remains an open question.

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Serum Erythropoietin Activity Following Kidney Transplantation

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31 patients with successful kidney grafts were studied. Ep, Ht, Hb and T lymphocytes were determined. Native and grafted kidneys were studied by ultrasonography. After KT, 45% of patients had PTE and in 71% of these a spontaneous regression was observed. Mean serum Ep activity in patients with and without PTE was significanthly higher than in healthy controls. Different erythroid colony growth sensibility and responsiveness to higher serum Ep (PTE and non-PTE patients) may be due to T3 cell interaction with BFU-E.

Keywords: erythropoietin, erythrocytosis, kidney transplant

Introduction

Erythrocytosis, a common late complication in patients after successful kidney transplantation (KT) has been previously reported [1, 2]. Moreover, in these patients an elevated peripheral erythropoietin (Ep) concentration was also found [2]. To elucidate the role of Ep in kidney transplanted patients, we determined serum Ep levels and performed a retrospective analysis of factors that may influence the development of post-transplant erythrocytosis (PTE). Furthermore, in all patients the ultrasonography of native and grafted kidney was performed.

Materials and Methods

31 patients (18 males, 13 females; age 41 $\pm$ 9, range 24–57 years) with successful grafts were studied. Ep, Ht, Hb and T lymphocytes were determined from blood samples.

For the evaluation of the Ep level, we analyzed the capacity of studied serum to stimulate labelled iron uptake in Guinea pig bone marrow cultures according to the method of Goldwasser et al. [3]. Bone marrow cell suspensions (10–15 $\times$ 10^6 nucleated cells/ml) from femurs of 3–4 months old Guinea pigs

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were cultured in a medium of 65% NCTC 109 containing 30% fetal calf serum, 5% human test serum and penicillin-streptomycin (100 U/ml and 100 μg/ml). A volume of 200 μl of the cellular suspension was placed in sterile microtiter plates and incubated for 20 hours at 37 °C in a humidified 5% CO_2-air incubator. Hereafter, 10 μl of a solution containing 50% normal AB human serum, 40% NCTC 109, 5% 0.9 M $NaHCO_3$ and 5% ^{59}Fe was added to each microtiter well and plates reincubated for 5 hrs. Cells were removed, collected into glass tubes, washed and heme was extracted using acid ethylmethylketone. ^{59}Fe incorporation into heme was determined by measuring radioactivity in aliquots of the solvent layer in a gamma-counter. Ep level was calculated by the following formula [4]:

$$\% \text{ Ep level} = \frac{\text{cmp patients serum}}{\text{cpm normal AB serum}} \times 100$$

The pooled AB sera of 30 normal subjects was used to determine the control values of Ep.

T cells were determined using murine monoclonal antibodies directed against pan-T cells (OKT3).

Table 1

Clinical aspects and kidney serum Ep activity in 31 transplanted patients

	PTE-Patients	Non-PTE patients	Controls
– Mean time (months) between KT and Ep measurement	66 ± 39 (8 – 100)	43 ± 34 (4 – 104)	–
– Mean serum erythropoietin (% of control)	*160 ± 30 (120 – 200)	*160 ± 20 (130 – 190)	100 ± 15
– Mean azathioprine dose (mg/kg body wt)	2.0 ± 5	2.2 ± 7	–
– Spontaneous regression of erythrocytosis (1–68 months)	64%	–	–
– Intermittent phlebotomy (Ht 57 ± 6)	36%	–	–
– Hypertension	100%	90%	–
– Transplant artery stenosis	–	6%	–
– Active hepatitis	–	9.6%	–

* $P < 0.005$ vs controls
PTE = post-transplant erythrocytosis
KT = kidney transplantation

Ultrasonography: commercially available real time sector scanners with 3.5 MHz transducers were used (AUC 940, Ansaldo).

Results were evaluated by Students' unpaired *t*-test and simple linear regression analysis.

Results and Discussion

In 45% of patients PTE (Ht = 52 ± 4%) with normal white cell and platelet count was found at different times after KT (average 7 ± 3 months). In patients without PTE (Ht = 48%), the normalization of Ht was average 4 ± 1 months [5].

Spontaneous regression of PTE occured in 71% of the patients. Intermittent phlebothomy (Ht = 57 ± 6%) was performed in 4 patients to prevent thromboembolic events.

Mean serum Ep concentration (Table 1) was significantly higher ($P < 0.001$) in 63% of all patients (with or without PTE) than serum Ep titers in healthy controls. There was no significant difference between the two studied groups of patients (with or without PTE) concerning mean azathioprine dose [6], trans-

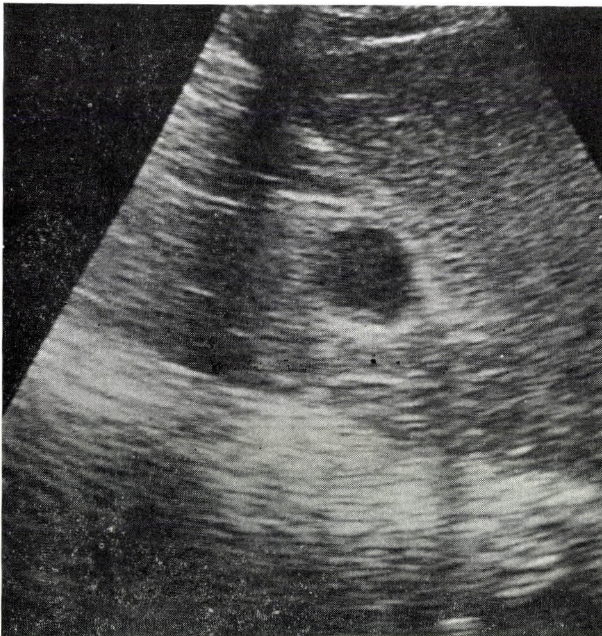

Fig. 1. Native kidney (dx) ultrasound, showing fibrotic alterations and acquired cystic disease in patient with high Ep (15 yrs. from HD start; 10 yrs. post KT)

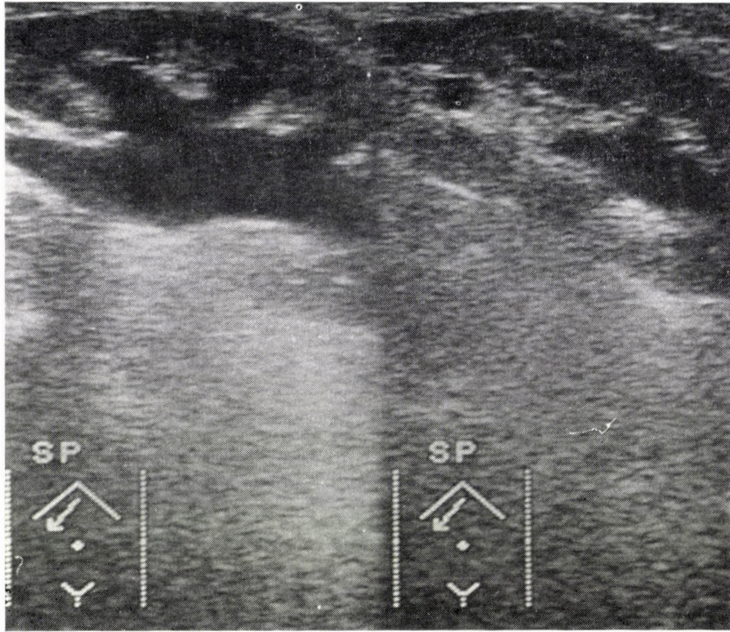

Fig. 2. Graft ultrasound, showing moderate hydronephrosis in patient with high Ep (13 months post KT)

plant artery stenosis, severe hypertension and active hepatitis (Table 1), implicated causes of erythrocytosis. A relationship between OKT3 count (%) and Ht was found statistically significant ($n = 20$; $r = 0.466$; $P = < 0.05$), while no correlation was found between Ht and total white cell counts. In all patients ultrasound pictures of native kidneys showed fibrotic alterations; in some cases acquired cystic disease was found. (Fig. 1). In one patient a moderate graft hydronephrosis was discovered. (Fig. 2).

The above mentioned anatomical conditions of the native kidneys may produce a persistent local hypoxia that leads to an increased Ep production according to studies by Thevenod et al. [5].

In conclusion, our results confirm that the increase of Ep production may be due to changes in the native kidneys. Only 35% of patients with high EP levels had PTE.

Different erythroid colony growth sensibility and responsiveness to higher serum Ep may be due to T3 cell interaction with BFU-E, as reported by Lampert et al. [7] and suggested by the correlation found between Ht and T3 cells.

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Chemotherapy of Adult Acute Nonlymphoblastic Leukaemia

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Seventy-two consecutive and previously untreated adults with acute nonlymphoblastic leukaemia (ANLL), having a median age of 36 years (range 12 to 71), were prospectively randomised to receive conventional doses of cytosine arabinoside and doxorubicin combined with either etoposide (CTR III) or 6-thioguanine (DAT). Morbidity was comparable between the two regimens and complete remission (CR) rates of 52% and 62% respectively ($p > 0.50$) were not influenced by age above or below 50 years, initial white cell count, French-American-British classification, or race. However, growth pattern in the GM : CFUc assay was found to identify a subgroup of patients who had a significantly higher CR rate. Similarly, the secretion of tissue plasminogen activator by leukaemic blasts in vitro uniformly predicted for primary drug resistance, whereas a CR rate of 68% was associated with production of the urokinase type or a mixture of both enzymes. Remission duration and survival did not differ between these two forms of chemotherapy, nor were they influenced by immunotherapy with C. parvum or the duration of maintenance therapy, whereas age below 50 and the species of plasminogen activator secreted were significant prognostic factors. It is concluded that etoposide can be substituted for 6-thioguanine in these cytosine arabinoside and doxorubicin-containing regimens and that for both combinations the most sensitive prognostic factor for CR and survival is the species of plasminogen activator secreted in vitro by the leukaemic blasts.

Keywords: etoposide, DAT, GM : CFUc, TPA secretion

Introduction

Optimal survival in acute nonlymphoblastic leukaemia (ANLL) depends upon achieving the highest possible complete remission (CR) rate combined with prevention of subsequent relapse. The most effective induction regimens contain cytosine arabinoside and an anthracycline antibiotic [1]. Etoposide, an epipodophyllotoxin designated VP16-213, has been shown to be a useful agent in the treatment of ANLL [2, 3] and in previous studies this agent has been successfully combined with cytosine arabinoside and an anthracycline antibiotic [4, 5]. Al-

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though many regimens contain additional drugs, any benefit so derived remains to be documented [6, 7]. 6-thioguanine has been advocated in this context and at the time this study was initiated a 5-day course of these three agents, designated DAT, was reported to have a CR rate of 85% [8]; this figure has, in subsequent larger studies, dropped to 66% [9]. The current study was designed to prospectively compare the replacement of 6-thioguanine by etoposide as a third drug in the treatment of ANLL. Two additional aims of the present investigation were to extend our experience with the duration of maintenance therapy [4] and to evaluate the benefit of C. parvum as a form of immunotherapy [10].

Materials and Methods

Patients

Seventy-two adults were entered into this study between March, 1981 and January, 1985; the protocol had received approval by institutional review boards. Informed consent was required and all those who completed the first course of chemotherapy were considered evaluable. Patients with acute leukaemia secondary to the myelodysplastic syndrome or who had received previous chemotherapy or radiotherapy were excluded.

Laboratory studies

All patients were classified according to the French-American-British recommendation [11], based on examination of peripheral blood and bone marrow aspiration. At presentation, additional studies included full blood count, biochemical profile, in vitro bone marrow culture for GM : CFUc [12], and characterisation of the type of plasminogen activator produced by leukaemic cells [13, 14]; these were repeated serially, where appropriate.

Immunotherapy was assessed by serial in vitro studies of the peripheral blood mononuclear cell fraction, isolated on a ficoll : hypaque gradient (specific gravity 1.077 g/ml) from both control patients and those receiving C. parvum. Under standardised conditions 5×10^5 mononuclear cells/ml were suspended in RPMI tissue culture medium (Gibco Limited, Scotland) containing 25% heat inactivated human pooled AB serum in the presence of three different mitogens: concanavalin A (Calbiochem Behring Corporation, Hoechst, Lucerne, Switzerland), 250 μg/ml; phytohaemagglutinin (Wellcome, Dartford, U.K.), 450 μg/ml; and pokeweed (Flow Laboratories, Herts, U.K.), 20 μg/ml [15]. Following 3 days' incubation at 37 °C, each well was pulsed for 18 hours with 1 uCi tritiated thymidine (Amersham Radiochemical Centre, U.K.) and, after harvesting, radionuclide incorporation was determined by liquid scintillation counting (Tricarb Model C2425-Packard, Downers Grove, Illinois, U.S.A.). Simultaneous studies

were carried out using the mixed lymphocyte reaction [16], in which 5×10^5 mononuclear cells/ml were exposed to alloantigen in the form of an equal number of mitomycin treated lymphocytes for 5 days, pulsed with tritiated thymidine, and a stimulation index established by scintillation counting. All studies were carried out in triplicate and responses expressed as a percentage of pretreatment values.

Supportive care

Patients were treated in general medical wards, without prophylactic antibiotics. With the development of sustained fever, appropriate blood cultures were taken and the patients started on a combination of broad spectrum antimicrobial therapy. Packed red cells were transfused to maintain the haemoglobin above 12 g/dl. Platelet transfusions were used for thrombocytopenic bleeding and allogeneic granulocytes infused in patients with unresponsive infections.

Chemotherapy

Patients were randomly assigned to receive a 3-drug combination regimen (CTR III) comprising cytosine arabinoside (80 mg/m^2 by continuous intravenous infusion on days 1 through 5), doxorubicin (50 mg/m^2 on day 1) and etoposide (80 mg/m^2 on days 1 through 5 as a half-hour infusion). The alternative 3-drug regimen (DAT) consisted of cytosine arabinoside (100 mg/m^2 every 12 hours on days 1 through 5 as a half-hour intravenous infusion), doxorubicin (50 mg/m^2 on day 1), and 6-thioguanine (100 mg/m^2 orally every 12 hours on days 1 through 5).

Patients with persistent leukaemia on day 21 after initiating therapy were designated as treatment failures and a second course of induction therapy was commenced. Up to four such cycles were given before they were regarded as having refractory disease and removed from the study. Consolidation therapy consisted of two further monthly courses of the same drugs, given immediately after complete remission (CR) had been achieved.

Patients in CR were randomised to either the short (6 months) or extended (15 months) arm of the maintenance programme. In the short course, cyclophosphamide was infused intravenously once in each of three consecutive months, starting at a dose of 1250 mg/m^2 and escalating this to achieve a nadir of granulocyte count below 1.5×10^9/l and platelet count below 50×10^9/l. Thereafter, three different drugs were administered monthly for 3 months. Methotrexate was given in a loading dose of 0.5 mg/kg over 30 minutes, followed by a 24 hour infusion of 125 mg/m^2, after which the patient underwent folinic acid rescue. In the same 24 hours 2 mg of vincristine was infused. On the second day 250 mg/m^2 of cytosine arabinoside was infused over 24 hours and the dose titrated in the second and third courses to achieve granulocyte and platelet counts comparable to those obtained during the cyclophosphamide infusion. In the extended course

there were also two phases. In each of the first 9 months a 24 hour course of chemotherapy was given, comprising continuous infusion of cytosine arabinoside (400 mg/m^2), etoposide (400 mg/m^2) in five divided doses, and doxorubicin (50 mg/ /m^2) given as a single injection. The dose of cytosine arabinoside was again adjusted to achieve the desired suppression of granulocyte and platelet counts. In the ensuing 6 months the patients received identical chemotherapy to that given during the short course. Patients were randomly allocated to receive C. parvum (Corpovax, Burroughs Wellcome, England), 2.5 mg once a week by intravenous infusion throughout the period of induction, consolidation and maintenance.

Surveillance

Bone marrow examination was performed monthly during treatment and every 3 months after completion.

Definition of therapeutic endpoints

Complete remission was defined as less than 5% blasts in a normocellular marrow, with return of peripheral blood counts to normal. Relapse was defined as the appearance of circulating leukaemic blasts or greater than 5% blasts in the bone marrow.

Statistical analysis

Survival duration was measured from the day of the first treatment to the day of death. Remission duration was measured from the time of detection of bone marrow remission to the date of relapse. The probability of staying in remission or of being alive was calculated according to the Kaplan–Meier method [17]. Response rates were compared using the chi-squared test with Yates' continuity correction or Fisher's exact test for small numbers. Remission and survival curves were compared by using the Generalised Wilcoxon and the Mantel–-Cox tests, employing the BMDP-1L computer program.

Results

Remission induction chemotherapy

Thirty four patients were randomised to receive CTR III and 38 to receive DAT, but two were subsequently excluded from analysis due to major protocol violations. Age, sex distribution, FAB subtype and white cell count were not statistically different between the two treatment groups (Table 1). The CR rate

of 52% (17/33) achieved in patients receiving the CTR III regimen was not significantly different from the 62% (23/37) in those who received DAT ($p > 0.05$). Of the pretreatment characteristics analysed for power to predict response rate there was no significant influence by age, pretreatment white cell count, FAB subtype, or race.

Table 1

Characteristics of the two treatment groups

	CTR III	DAT
Number	33	37
*Age (Years)		
— Median/Mean	45/38.1	31/34.0
— Range	12—68	17—71
Males	20	17
Females	13	20
FAB M1	14	15
M2	10	13
M3	1	1
M4	6	6
M5	2	2
**White cell count ($\times 10^9$/1)		
— Median/Mean	12.5/34.9	12.9/30.3
— Range	2.2—200.0	0.4—238.0

* $p = 0.3$; ** $p = 0.69$ } Student's *t*-test.

Table 2

Predictive value of growth pattern in the GM: CFUc assay

Type of growth pattern	Number of patients: Total	Number of patients: Achieving CR	% CR
1	18	12	67
2	23	11	48
3	7	7	100

Type 1 = No growth; Type 2 = Clusters only; Type 3 = Clusters and colonies; Clusters equal aggregate containing less than 50 cells each. Colonies equal aggregate containing more than 50 cells each.

Chi-square = 6.446, d.f. = 2, $p = 0.039$; Comparing "2" and "3" Fisher's $p = 0.015$; Comparing "1" and "3" Fisher's $p = 0.10$; Comparing "1" and "2" Fisher's $p = 0.18$

Table 3

Predictive value of plasminogen activator for complete remisson

Type of plasminogen activator	Number of patients		CR (%)
	Total	Achieving CR	
Tissue	10	0	0
Mixed or urokinase	53	36	68

Chi-square = 13.9, p = 0.0002
Fisher's p = 0.00007

In vitro bone marrow assay for GM : CFUc was performed on samples aspirated from 48 patients and the results grouped according to the growth pattern observed (Table 2). Those individuals where no growth was obtained (Type 1) had a CR rate of 67%; this is not statistically significantly different from the CR rate of 48% which was achieved by those patients where cluster formation alone, defined as cell aggregates of less than 50 in number (Type 2), was found. In contrast, those patients whose marrow cultures gave rise to both clusters and colonies, the latter defined as more than 50 cells in an aggregate (Type 3), had a significantly superior CR rate of 100% ($p = 0.039$).

The species of plasminogen activator secreted by the leukaemic blasts was studied in 63 patients. In those individuals where predominantly tissue type enzyme was found none achieved CR, whereas 68% of those patients whose cells secreted predominantly urokinase or a mixture of both types of plasminogen activator achieved this status ($p > 0.001$) (Table 3).

Remission failure

Thirty patients failed to achieve a CR. The pattern of remission failure [18] is shown in Table 4. The CTR III regimen was associated with more Type V treatment failures than occurred with DAT (Fisher's $p = 0.04$).

Remission duration

The median durations of CR were 27.5 weeks and 30 weeks for the CTR III and the DAT regimens, respectively. There was no significant difference in remission duration when these two regimens were used as induction and consolidation therapy (Fig. 1). The species of plasminogen activator secreted by the leukaemic blasts was found to influence the duration of remission in those patients on these cells when such studies were performed. Where this was predominantly urokinase, remissions were significantly longer than when a mixed pattern

Table 4

Pattern of remission failure

Type of remission failure	CTR III	DAT
I	1	3
II	7	8
III	0	0
IV	0	1
V	8*	2

* Fisher's p = 0.04

was found (p = 0.04; Fig. 2). For the entire group only 3 patients remain in remission.

Maintenance therapy

Thirty-two patients were randomised to the maintenance programme. Thirteen patients received the short course and 19 patients the extended course. Eight patients who achieved CR did not receive maintenance therapy; 2 patients underwent bone marrow transplantation, 2 refused maintenance chemotherapy, and 4 patients died while aplastic during consolidation therapy. Analysis of the data revealed no statistically significant difference in remission duration, being 24 weeks for the short course and 35 weeks for the extended course when analysed regardless of the induction regimen. Patient numbers were too small to examine

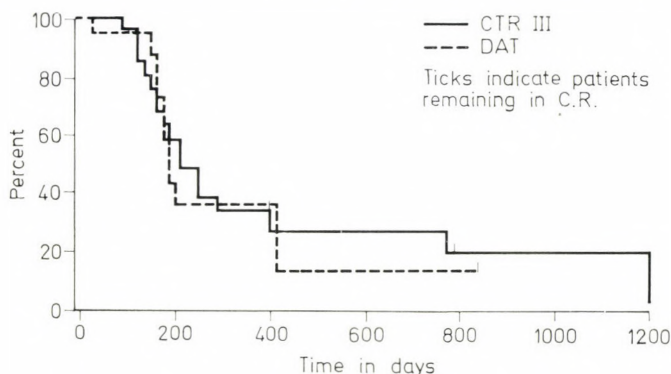

Fig. 1. Comparison of the two induction regimens. Kaplan–Meier plot of cumulative proportion of patients surviving in remission. CTR III is cytosine arabinoside, doxorubicin and etoposide and DAT is cytosine arabinoside, doxorubicin and 6-thioguanine. Ticks indicate patients remaining in complete remission

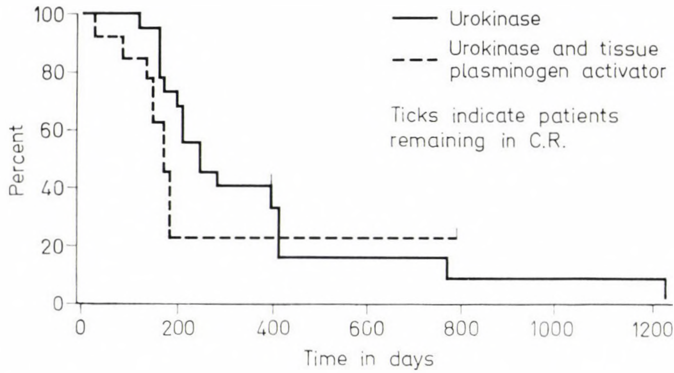

Fig. 2. The influence of plasminogen activator secretion on survival. Kaplan–Meier plot of cumulative proportion of patients surviving in remission. Ticks indicate patients remaining in complete remission. One patient who is alive in CR is not shown on this graph since these studies were not carried out in that individual

separately the effect of maintenance therapy in conjunction with the type of induction regimen used.

Immunotherapy

Twenty-nine patients were randomised to receive C. parvum, commencing with the initiation of induction chemotherapy. This agent had no significant influence on the CR rate, duration of remission or survival.

The in vitro studies of peripheral blood mononuclear cell response to mitogens or alloantigen showed no change from pretreatment levels in patients receiving C. parvum. Furthermore, using the same laboratory techniques, serial studies performed throughout the induction, consolidation and maintenance phases of

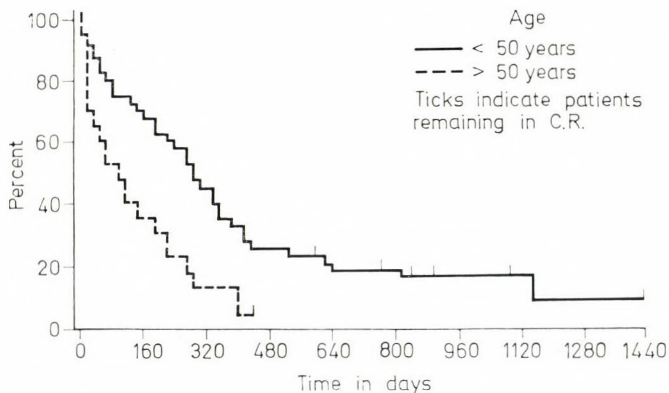

Fig. 3. The influence of age on survival. Kaplan–Meier plot of cumulative proportion of patients surviving in remission. Ticks indicate patients remaining alive

both chemotherapy programmes showed no differences between those patients who did or did not receive C. parvum. Similarly, when serial GM : CFUc assays were carried out on these patients no effect was demonstrable.

Survival

Median duration of survival for all patients treated with CTR III was 27 weeks; this is not significantly different from the 34 weeks achieved with those receiving DAT. Patients over the age of 50 were shown to have a significantly shortened duration of survival ($p = 0.003$) (Fig. 3). The survival curves have not reached a plateau.

Discussion

Several studies have shown that treatment of acute nonlymphoblastic leukaemia is most effective with regimens containing cytosine arabinoside and an anthracycline antibiotic [1]. The reported remission rates vary widely, ranging from less than 50% to greater than 80% [8, 9, 19, 20] as a result of many factors, of which the most important is age [19, 21, 22] but which also includes other variables such as the level of expertise and supportive care available.

At the time the present study was initiated the best combination of chemotherapy had not been clearly defined. In at least two small series of patients a CR rate exceeding 80% was achieved with regimens that included 6-thioguanine (DAT) [8, 20]. As our previous experience had been gained using etoposide in combination with cytosine arabinoside and an anthracycline antibiotic [4, 5, 23] it was decided to prospectively evaluate this programme in comparison to DAT. These regimens differ, however, in two ways. Firstly, the cytosine arabinoside in CTR III is given as a continuous intravenous infusion, whereas in DAT, bolus injections are given at 12-hourly intervals. Secondly, in CTR III, etoposide replaces the 6-thioguanine used in DAT. Although the CR rate achieved with DAT was marginally higher than with CTR III this difference was not statistically significant. Furthermore, the duration of remission and survival with both regimens is similar. It is noteworthy that in this study the median duration of remission in patients receiving DAT was only 30 weeks in contrast to the 64 weeks reported by the British Medical Research Council, based on results obtained by a number of centres using a comparable regimen [9]. It is difficult to explain this difference other than by chance occurrence.

Type 1 and 2 remission induction failures for both chemotherapeutic regimens are comparable, suggesting equal anti-leukaemic efficacy. Type V failures were statistically greater with CTR III, but since the number of patients in these groups is relatively small, this difference may be fortuitous, particularly as supportive care was uniform. It is difficult to comment about toxicity because these patients died within 7 days of completing chemotherapy and could not be reliably evaluated [18].

Subsequent to the commencement of the present study, a number of reports have appeared suggesting that a more intensive regimen, in which cytosine arabinoside is extended to 7 or even 10 days by continuous intravenous infusion and an anthracycline antibiotic is overlapped for the first 3 days, may be a more effective approach than the lesser chemotherapy given in programmes such as CTR III and DAT [19, 24–26]. It is, however, noteworthy that when a large number of patients are studied, the overall complete remission rate for these programmes is 53% and extending the duration of cytosine arabinoside from 7 to 10 days confers no additional benefit [19]. The main advantage of a more intensive induction and consolidation regimen may be evident, not so much in an improved CR rate as in the duration of remission, particularly in those patients who remain relapse-free for 5 years or more and where the survival curves have reached a plateau, indicating a higher probability of cure. Of the 40 patients in the present study who achieved CR 3 remain relapse free at 58+, 115+ and 121+ weeks. Follow-up is still short, but these results are disappointing in terms of cure rates for leukaemia using chemotherapy alone, where more intensive combination drug programmes have achieved 15% to 25% long term disease-free survival [24, 27]. We have therefore intensified the chemotherapy used for induction and consolidation, giving 7 days of both cytosine arabinoside and etoposide combined with 3 days of an anthracycline antibiotic, as has been employed by others [3].

The role of maintenance therapy in acute nonlymphoblastic leukaemia remains controversial [1] and there are reports that suggest this is without benefit [26], although conflicting studies in support of postremission therapy have been published [28–30]. Here again, there is evidence that when large numbers of patients are studied no benefit accrues from extended periods of maintenance [19]. In the present study it was not possible to examine the basic issue of efficacy of maintenance therapy since there was no control group that was denied post-remission treatment, but we are able to comment on the question of duration, chemotherapeutic agents and schedule in this context. In an extension of a previous study [4], we have been unable to show an advantage for either remission duration or survival following a longer course of maintenance therapy. It seems probable that for therapy to be effective when administered after consolidation it would need to be intensive if residual leukaemic cells are to be eliminated. The present experience would suggest that the maintenance programmes employed in this study may well have been ineffective. Accordingly, our current practice is to administer high doses of chemotherapy, with or without additional radiotherapy, followed by allogeneic or autologous bone marrow transplantation as a better approach to post-remission therapy aimed at extending the duration of disease-free survival [31].

Of the prognostic factors evaluated, the present study confirms the sensitivity of the species of plasminogen activator secreted by leukaemic blasts in predicting response [14, 32] to these less intensive chemotherapy programmes. It remains to be established whether this observation holds true when chemotherapy is intensified. Similarly, the in vitro growth pattern, using the GM : CFUc

assay [33, 34], may be an independent variable predicting for response and further studies are needed to standardise the criteria used in reporting these results. Age [19, 21, 22] is said to be a significant factor determining response to chemotherapy and we were able to demonstrate an effect of this variable on the duration of survival. Of the other prognostic factors described, no influence could be demonstrated in terms of the pretreatment white cell count [35, 36], the FAB classification [37] or race [38, 39].

Immunotherapy, including the use of C. parvum, has been shown in many trials to be of little or no benefit in the treatment of acute nonlymphoblastic leukaemia [40–42]. The administration of this agent was based on studies suggesting that it is active as an immunotherapeutic anticancer agent [43] and has T-cell dependent activities in tumour systems [44], as well as having an effect on colony stimulating factor [45, 46]. However, the infusion of C. parvum is known to have significant side effects [47, 48] and in our experience patient acceptance of the immunotherapy was poor, being frequently associated with rigors and pyrexial reactions which could not always be blocked pharmacologically. In keeping with previous reports, we have been unable to show any benefit in remission rate, duration or survival of the patients receiving C. parvum. In addition, serial laboratory studies on the effect of mitogens and alloantigen on the mononuclear cell population harvested from patients receiving immunotherapy showed no difference from control values. Similarly, C. parvum administration to patients was found to be without effect on the in vitro GM : CFUc assay.

It is concluded that the substitution of etoposide (CTR III) for 6-thioguanine (DAT) in a chemotherapy regimen containing 5 days of cytosine arabinoside and a single injection of doxorubicin has no significant advantage. The question remains unanswered as to the benefit that may accrue when either of these agents is added to programmes containing cytosine arabinoside and anthracycline antibiotic. We have demonstrated no benefit in extending the duration of maintenance chemotherapy or the use of C. parvum as a form of immunotherapy in the treatment of adult patients with acute nonlymphoblastic leukaemia.

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Combination Chemotherapy for Advanced Diffuse Large Cell Lymphoma. The Adverse Effects of Bone Marrow Invasion, Gastrointestinal Tract Involvement or High Bulk Disease

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Thirty-nine adults with clinical stage III or IV diffuse large cell lymphoma were prospectively randomised to receive etoposide with doxorubicin (Group 1: n = 17), the same schedule of etoposide with carminomycin (Group 2: n = 8), or BACOP (Group 3: n = 14). The complete remission rates were respectively 24%, 25% and 28%, and further good partial remissions were 41%, 25% and 14%. The incidence of adverse prognostic factors was examined with the first two groups combined for comparison to patients receiving BACOP. The low complete remission rates were attributable to bone marrow invasion in 64% (16/25) of patients in groups 1 and 2, and 64% (9/14) in group 3; to extensive gastrointestinal tract involvement in 24% (6/25) of patients in groups 1 and 2, and 36% (5/14) in group 3; and to high bulk disease in 24% (6/25) of patients in groups 1 and 2, and 36% (5/14) in group 3. Actuarially predicted survival has not been reached for group 1, is 12 months for group 2, and 8 months for group 3; these different trends are not statistically significant. The trial was discontinued when it became clear that there was no difference between the two- and five-drug treatment regimens and that unacceptably low remission rates were obtained in patients having a high incidence of these poor prognostic factors, particularly when compared with results being reported in regimens that contain high or intermediate doses of methotrexate.

Keywords: etoposide, doxorubicin, carminomycin, BACOP

Introduction

Late stage diffuse large cell lymphoma follows a rapidly progressive clinical course when untreated [1] and, while early attempts at chemotherapy were relatively ineffective, a significant cure rate was reported with combination chemo-

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therapy [2]. This important observation has subsequently been confirmed and similar results achieved with comparable multi-drug programmes such as BACOP [3] and C-MOPP [4] regimens, where complete remission rates where in the region of 45%. It is relevant that only 5% of patients with stage III and IV disease achieved complete remission with single agent chemotherapy [5], but superior results were obtained with etoposide, when this drug was used either alone or in combination with only an anthracycline antibiotic, when remission rates were found to match those for the BACOP and C-MOPP regimens [6, 7]. However, patients treated with the latter regimens showed a superior disease-free remission rate and survival curve when compared to those who received etoposide and doxorubicin. To determine whether these differences could be ascribed to inferiority of the two-drug regimen or whether different institutional practice or patient population could influence the outcome of therapy, direct comparison was undertaken between the two regimens, paying particular attention to prognostic factors known to influence response rates [4, 8].

Materials and Methods

Thirty-nine patients with clinical stage III and IV diffuse large cell lymphoma [4, 8] were prospectively entered on a trial and randomised using a computer generated programme to compare etoposide (60 mg/m^2 as a 1 hour intravenous infusion on 5 consecutive days) and doxorubicin (40 mg/m^2 over 5 minutes on day 1), followed by a 9 day rest period (Group 1: n = 17) to the same schedule of etoposide with carminomycin (20 mg/m^2 over 5 minutes by intravenous infusion on day 1), also followed by a 9 day rest period (Group 2: n = 8) or BACOP (Group 3: n = 14): in all three groups responding patients received 8 cycles of therapy. The trial was approved by institutional review boards and entry required informed consent.

All patients had a history taken and underwent full physical examination, after which they were clinically staged. Laboratory investigations included full blood count, bone marrow aspiration and trephine biopsy [9], biochemical profile [10], chest radiology and bipedal ascending lymphangiography or computerised axial tomography. Lymph node biopsy demonstrated diffuse large cell lymphoma [11] and immunophenotyping [12] showed these to be B-cell tumours. Prognostic factors were carefully documented in each patient, using published criteria [4, 8], with emphasis on bone marrow invasion, gastrointestinal tract involvement, or the presence of high bulk disease.

The side effects of the chemotherapy were limited to alopecia in groups 1 and 2, and variable pancytopenia in all three groups, leading to appropriate dosage adjustment with each cycle of chemotherapy.

Results

The age and sex distribution of the patients is given in Table 1.

It is noteworthy that in this study there was a large proportion of individuals with biopsy proven marrow invasion, gastrointestinal tract involvement, or high bulk disease (Tables 2a and 2b).

The response rates for the patients were defined using established criteria [13]: the complete remission rate for Group 1 was 24%, for Group 2 was 25%, and for Group 3 was 28%, with further good partial remissions in 41%, 25% and 14%, respectively (Table 3): the median duration of these complete remissions

Table 1

Demographic details of patients

	Group 1 Etoposide and Doxorubicin	Group 2 Etoposide and Carminomycin	Group 3 BACOP
Male : Female	9 : 8	3 : 5	10 : 4
Median age	56 years	63.5 years	53 years
Range	18–70 years	29–71 years	29–77 years

Table 2

a) Prognostic factors in the initial three groups

Prognostic factors	Group 1	Group 2	Group 3
Bone marrow Positive	12/17 71%	4/8 50%	9/14 64%
Gastrointestinal Involvement	3/17 18%	3/8 38%	5/14 36%
High bulk disease	4/17 24%	2/8 25%	5/14 36%

b) Prognostic factors combining groups 1 and 2

Prognostic factors	Groups 1 and 2	Group 3
Bone marrow Positive	16/25 64%	9/14 64%
Gastrointestinal involvement	6/25 24%	5/14 36%
High bulk disease	6/25 24%	5/14 36%

Groups 1 and 2 were pooled for this analysis as remission rates for the two anthracycline-containing arms were indistinguishable.

Table 3

Analysis of remission rates and survival at median follow-up of 36 months

	Group 1 Etoposide and Doxorubicin	Group 2 Etoposide and Carminomycin	Group 3 BACOP
Alive	10/17 59%	2/8 25%	4/14 28%
Early deaths	2/17 12%	1/8 12.5%	1/14 7%
Partial remission	7/17 41%	2/8 25%	2/14 14%
Complete remission	4/17 24%	2/8 25%	4/14 28%

was 18 months, 3 months and 33 months. These numbers are too small for reliable statistical analysis.

Withdrawal of carminomycin from the market unfortunately prevented entry of further patients to this arm of the study. The actuarially predicted survival rate has not been reached for group 1, is 12 months for group 2, and 8 months for group 3: despite this trend in favour of group 1, there is no statistically significant difference between any of these curves (Figure 1). However, the relatively small number of patients entered before the study was terminated on ethical grounds do not yield comparisons with sufficient power to exclude anything other than major differences.

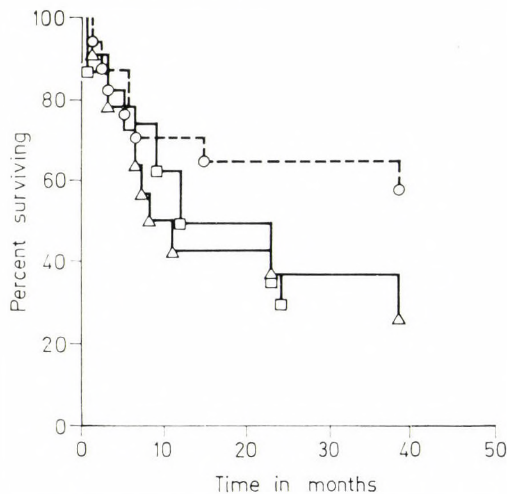

Fig. 1. Actuarially predicted survival curve. The median survival has not been reached in patients who received etoposide and doxorubicin (Group 1; n = 17) (black dots), is 12 months for those receiving etoposide and carminomycin (Group 2; n = 8) (black triangles), and 8 months for BACOP (Group 3; n = 14) (white squares)

Results and Discussion

It has previously been demonstrated [6, 7] that etoposide has activity in diffuse large cell lymphoma when used on its own. Furthermore, when this agent was combined with doxorubicin the remission rate improved markedly and then approximated that obtained with the C-MOPP and BACOP programmes [4, 8].

In the present study an equivalent complete remission rate was again found when BACOP was directly compared to etoposide with either of the anthracycline antibiotics. However, these figures are lower than in our previous studies, now attributable to an unusually high incidence of poor prognostic factors, with the latter being uniformly distributed throughout the patient population. The explanation for this finding is not clear, but it does emphasize two points. Firstly, there is the question of historical controls, even within a single institution, since, unless very large numbers of patients are entered into each study, erroneous conclusions may result when data from consecutive series are compared. This is exemplified by the difference in survival curves for our two groups of patients receiving etoposide and doxorubicin, despite there being no reason to believe that the overall referral pattern had in any way changed. However, the number of individuals with poor prognostic factors was much greater in the present series and had comparison been attempted between these two consecutive trials the discrepant results might have been more difficult to explain. On the other hand, randomisation within a single study has corrected for this unexplained preponderance of adverse features and revealed a close similarity in response to all three treatment options. Secondly, it might be anticipated that an even greater disparity would exist between patient populations at different institutions, a point previously pointed out [7]. This latter point is evident when an American [4, 8] and South African [6, 7] experience are compared. Thus, the remission rate for both groups using comparable chemotherapy is similar, whereas the American patients have a higher stable disease-free plateau, suggesting that the greater incidence of poor prognostic factors that characterises the South African patients adversely affect the durability of their remission status. Failure to appreciate this fundamental difference between the two patient populations might therefore have legitimately raised a question about the combination of etoposide with doxorubicin being less effective than BACOP.

Another feature of the current series is the frequent co-existence of poor prognostic factors. In only 6 of the 39 patients (15%) was either extensive gastrointestinal tract involvement or high bulk disease present without unequivocal marrow involvement on the trephine biopsy. Unfortunately, in view of the limited number of patients available, it was not possible to reliably determine whether a negative marrow in the presence of the other poor prognostic features had a similar adverse effect on response to therapy.

A further point of interest is the survival of the patients who received etoposide with doxorubicin (group 1). Although only 4/17 (24%) achieved complete

remission, 10/17 (59%) were alive at the time of analysis, in contrast to groups 2 and 3 where a more usual outcome was found in which survival directly correlated with the achievement of complete remission. There is no obvious explanation for this unexpected finding since the patients in all three groups were generally well matched for the variables, but analysis shows that the 6 patients who achieved partial remission in group 1 had only minimal residual disease evident and each of these patients then responded to salvage therapy with BACOP and abdominal radiotherapy, although only 1 achieved a second complete remission.

In conclusion, it is emphasized that recent advances in chemotherapy have impressively raised remission rates and resulted in both improved disease-free duration and survival curves [7, 13–15]. Furthermore, it is clear that these more aggressive regimens are diminishing the value of traditional prognostic factors in predicting the outcome when patients were treated with older programmes such as C-MOPP and BACOP. Under these circumstances, this comparative study was terminated with the recognition that neither etoposide combined with an anthracycline antibiotic nor BACOP, despite their equivalence in achieving remission rate and similarity of survival curves, is appropriate for treating stage III and IV diffuse large cell lymphoma. This latter observation would be particularly pertinent in the type of population seen at our institution, where the majority of patients present with an unusually high prevalence of adverse prognostic factors. Finally, since the significant difference between BACOP and the clearly superior m-BACOD regimen apparently resides in the addition of methotrexate [16, 17] this latter programme is presently being directly compared to the previously described etoposide and doxorubicin combination to which an equivalent amount of methotrexate with folinic acid rescue have been added.

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Surface Phenotype and Immunoglobulin Levels in B-cell Chronic Lymphocytic Leukaemia

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The aim of the present study is to analyze the relationship between serum immunglobulin (Ig) levels and the immunological phenotype and the clinicohaematological features of B-cell chronic lymphocytic leukaemia (B-CLL) in a series of 126 patients. Eighty-eight of the cases (70%) had a decreased concentration of at least one Ig. IgM and IgA were the most frequently decreased (60% and 49% respectively). A serum monoclonal gammapathy was found in 4 patients, Ig M/k in two cases and IgM/1 and IgG/k in one case.

Patients with hypogammaglobulinaemia had a similar surface phenotype as patients with normal Ig levels (MRFC+, sIg+, CD20+, HLA/DR+, FMC7−, CD5+, CD9+). On the other hand the cases with hypogammaglobulinaemia displayed the features of a more advanced disease, a higher incidence of organomegalies ($p < 0.05$), anaemia and/or thrombopenia ($p < 0.05$), a diffuse bone marrow pattern ($p < 0.05$), advanced clinical stages ($p < 0.05$) as well as higher levels of both peripherial blood ($p < 0.02$) and bone marrow lymphocytosis ($p < 0.02$). These findings suggest that the presence of hypogammaglobulinaemia in B-cell patients is probably more related to a higher tumor burden than to either certain stages of B-cell differentiation or a particular cellular phenotype.

Keywords; surface phenotype, immunglobulin levels, B-CLL

Introduction

Chronic lymphocytic leukaemia (CLL) is a lymphoproliferative disorder, usually of B-cell origin [1]. A common finding as an alteration of the humoral response, furtheron a decreased immunglobulin (Ig) level in most patients and occasionally a monoclonal gammapathy, [2, 3, 4]. Most studies on Igs in CLL have focused in studying "in vitro" whether these alterations are due to an intrinsic B-cell defect or to abnormalities in T-cell subsets [5, 6, 7, 8, 9, 10, 11]. However, there are few studies in which attempts have been made to explore whether the serum Ig levels in B-CLL are related to certain stages of B-cell differentiation or whether they are related to some particular cellular phenotype.

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In the present study we analyzed the relationship between serum Ig levels and the immunological phenotype of neoplastic B-cells in 126 untreated B-CLL patients according to the presence or absence of hypogammaglobulinaemia.

Materials and Methods

The present study includes 126 untreated patients with the diagnosis of B--CLL according to clinicohaematologic criteria [12]: peripherial lymphocytosis ($>15\times10^9$ lymphocytes/l), bone marrow involvement ($>40\%$ lymphocytes), spontaneous mouse rosette-forming cells (MRFC) and/or surface Ig (sIg) positivity in more than 20% of cells. All patients were classified according to Rai's staging [12] and the International Workshop on CLL, staging systems [13]. Bone marrow biopsy was performed in 69 patients and four histopathologic patterns were considered according to Hernández-Nieto et al. [14].

Serum IgG, IgA and IgM levels were detected by nephelometry (Auto ICS Analyser II, Beckman Instrument). Hypogammaglobulinaemia was considered to exist when IgG levels were lower than 600 mg/dl and/or IgA lower than 120 mg/dl and/or IgM lower than 80 mg/dl [15].

Immunological studies: Mononuclear cells from peripheral blood (PB) were obtained by Ficoll-Hypaque (Pharmacia Fine Chemicals) density gradient centrifugation and tested for: a) spontaneous rosette-forming cells with AET-treated erythrocytes (SRFC); b) spontaneous rosette-forming cells with mouse erythrocytes (MRFC); c) surface immunglobulins (sIg) detected by direct immunfluorescence with rabbit F(ab)'2 antisera conjugated with fluorescein isothiocyanate (FITC); and d) surface antigens assessed by indirect immunofluorescence with monoclonal antibodies (McAb): GRB1 (anti class II MHC antigen) [16], B1 (anti p35 antigen or CD20) [16], Fmc7 (which recognises a subpopulation of normal PB B lymphocytes and the majority of cells in B-cell prolymphocytic leukaemia and hairy cell leukaemia) [17], Fmc56 (anti p24 antigen or CD9) [16], and Cris1 (anti p67 antigen or CD5) [16].

The percentage of MRFC+, sIg+, Fmc7+ and Cris1+ cells was referred to B lymphocytes after removing the percentage of SRFC-T cells.

To estimate the significance between means, the Mann-Whitney "U" test was used. A contrast for dichotomous variables was employed for binomial parameters (positive or negative cases).

Results

The mean values of each Ig in the current series were: IgG: 984 ± 557 mg/dl, IgA: 140 ± 90 mg/dl and IgM: 92 ± 94 mg/dl. Eighty-eight of 126 (70%) patients had a decreased concentration of at least one immunglobulin. IgM and IgA were the most frequently decreased (60% and 49% of cases, respectively). A simulta-

neous decrease of the three Igs was detected in only 13 patients (10%), while two Igs were diminished in 45 cases (36%). In this latter group the most common association was IgA and IgM (39 cases) followed by IgG and IgM (4 cases) and IgA and IgG (2 cases). Finally, 30 patients (23%) showed decreased levels of only one Ig: IgM in twenty, IgA in eight, and IgG in two patients.

Four patients showed a serum monoclonal gammapathy : IgM/k in two cases and IgM/1 and IgG/k in one case.

The clinical and haematologic characteristics of the patients with normal and low serum Ig levels are shown in Table 1. There was a greater proportion of accidentally discovered cases in the group of patients with normal Ig levels ($p < 0.1$) while the cases with hypogammaglobulinaemia showed a higher incidence of adenopathies ($p < 0.01$), hepatomegaly ($p < 0.05$), splenomegaly ($p < 0.05$), anaemia and thrombopenia ($p < 0.05$) as well as higher PB lymphocyte counts ($p < 0.02$). Moreover, patients with low serum Ig levels displayed a greater

Table 1

Clinical and haematological features of B-CLL patients according to serum immunoglobulin levels

	Normal Ig levels (N = 38)	Hypogamma-globulinaemia (N = 88)	p
Age*	67 ± 10	68 ± 10	n.s.
Male/female ratio	1.9	2.3	n.s.
Accidental discovery	55%	41%	< 0.1
Adenopathies	58%	72%	< 0.1
Splenomegaly	32%	48%	< 0.05
Hepatomegaly	18%	33%	< 0.05
Extralymphatic infiltration	3%	6%	n.s.
Anaemia (Hb < 10 g/dl)	11%	19%	n.s.
WBC count* (× 10⁹/l)	43 ± 41	76 ± 84	< 0.02
Thrombocytopenia (< 100⁹× 10/l)	8%	18%	< 0.05
LDH levels (IU/l)*	281 ± 110	287 ± 154	n.s.
Uric acid (mg/dl)*	6.0 ± 2.2	5.1 ± 1.6	n.s.
ESR (mm/h)*	28 ± 41	27 ± 34	n.s.
% of lymphocytes in BM*	65 ± 20%	74 ± 19%	< 0.02
Diffuse BM pattern	22%	43%	< 0.05
Second tumor	3%	10%	n.s.

* Results expressed as Mean ± SD

Table 2

Immunglobulin levels and clinical stages

	Normal Ig levels (n = 38)	Hypogamma-globulinaemia (n = 88)	p
RAI			
Stage 0	39%	20%	< 0.05
Stage I	21%	20%	n.s.
Stage II	24%	27%	n.s.
Stages III/IV	16%	33%	< 0.05
International Workshop			
Group A	63%	44%	< 0.05
Group B	21%	27%	n.s.
Group C	16%	29%	< 0.05

proportion of diffuse bone marrow pattern ($p < 0.05$) and a higher percentage of bone marrow lymphocytic infiltration ($p < 0.02$).

The distribution of patients according to the classification proposed by Rai et al. and the International Workshop is shown in Table 2. In both staging systems the cases with normal Ig levels included a significantly larger proportion of patients in early stages ($p < 0.05$). Conversely, there was a significantly higher incidence of advanced clinical stages within the group of patients with hypogammaglobulinaemia ($p < 0.05$). No differences were observed regarding the intermediate stages.

In 9 of the 88 patients (10%) with hypogammaglobulinaemia there was another malignancy associated with B-CLL; in cases of normal Ig levels this was found in only one patient (3%).

Comparing the patients with normal Ig levels to those with low ones, no significant differences were observed in the sIg heavy and light chain classes or in the expression of other surface antigens (Table 3).

The four patients with a serum monoclonal gammapathy expressed sIg of the $\mu + \delta +$ heavy chain class and in all cases the sIg light chain isotype was identical to the monoclonal serum Ig light chain: K in three cases and λ in one. These four patients expressed MRFC, sIg and reactivity for the McAbs: B1, Cris1 and GRB1 in more than 30% of B-cells. Positivity for FMC7 and FMC56 McAb was observed in one of these cases.

Discussion

Hypogammaglobulinaemia is one of the most commonly reported abnormalities associated with B-CLL [2, 3, 4]. Reduced concentrations of at least

Table 3

Relationship between surface markers and serum immunglobulin levels

	Normal Ig levels (n = 38)	Hypogammaglobulinaemia (n = 88)	p
SRFC	14 ± 14	11 ± 9	n.s.
MRFC	51 ± 24 (91%)	54 ± 25 (94%)	n.s.
sIg	50 ± 34 (83%)	53 ± 35 (64%)	n.s.
K/L ratio	1.6	2.9	n.s.
B1(CD20)	66 ± 26 (97%)	68 ± 27 (98%)	n.s.
GRB1(HLA-DR)	83 ± 21 (100%)	85 ± 19 (100%)	n.s.
FMC7	14 ± 23 (27%)	9 ± 20 (17%)	n.s.
Cris1(CD5)	46 ± 29 (83%)	50 ± 33 (82%)	n.s.
FMC56(CD9)	29 ± 29 (57%)	41 ± 35 (64%)	n.s.

Results expressed as mean ± standard deviation of positive cells. In brackets percentage of positive cases (> 10% positive cells)

one of the major serum Igs, primarily IgM, was observed in most of our cases. Patients with low serum Ig levels, also displayed features of a more advanced disease: a higher incidence of organomegalies, thrombopenia, an increased level of both peripheral blood and bone marrow lymphocytosis. In these patients a higher proportion of advanced clinical stages and a higher percentage of cases with diffuse bone marrow pattern were observed as also reported by other goups [3, 18, 19]. Such findings suggest that the presence of hypogammaglobulinaemia in B-CLL is probably related to a higher tumor burden and a greater replacement of normal B-cell populations by neoplastic lymphocytes. However, there is evidence that other factors may also be involved in the hypogammaglobulinaemia of B-CLL patients. The increase in supressor T-cell (CD8+) with a defective function of the helper T-cells (CD4+) reported in other series [20, 21, 22] also seem to contribute to the reduced serum Ig levels present in these cases.

It has been suggested that defective Ig production could be associated with a decrease in immunosurveillance and a higher incidence of neoplastic disease [23]. Moreover, it is well known that when compared to normal population of the same age, B-CLL patients display a higher proportion of other malignancies [24]. Analysing the incidence of a second tumor in our series, we observed that

patients with hypogammaglobulinaemia at the moment of diagnosis had a higher proportion of other neoplastic diseases compared to those B-CLL cases with normal serum Ig levels. B-CLL is the result of the monoclonal proliferation of small lymphocytes of B-cell origin. These leukaemic B-cells usually have sIg of either the μ + or μ + δ + heavy chain class as well as CD20, HLA-DR, CD9 and CD5 antigens. In addition, these cells form rosettes with mouse erythrocytes although they usually lack the FMC7 antigen [25, 26], as has also been found in the present series.

The serum Ig levels were found to be independent of the immunophenotype, which suggests that the presence of low serum Ig concentration is not correlated with a particular stage of B-cell maturation nor with a special phenotype in B-CLL.

Four of our 126 patients had a monoclonal component. In all cases the serum light chain was identical to the sIg light chain isotype expressed by the neoplastic B-cells. Other authors have also previously demonstrated that serum monoclonal proteins in B-CLL are closely related to the sIg of leukaemic B-cells [27]. The sIg heavy chain was of the μ + δ + isotype in these four patients, suggesting that cases with a detectable monoclonal serum Ig could represent the proliferation of a monoclonal B-cell at a more advanced stage of maturation [28] However, the expression of other differentiation antigens such as FMC7 [17, 29] was similar to that observed in the whole series. This finding, together with the results reported by Qian et al. [27], suggests that in such patients the neoplastic cell is arrested at a similar stage of maturation as the classical B-CLL although it displays a more incomplete blockage of Ig secretion.

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Whole Blood Suspension Culture Method for Chromosome Examination of a Patient with Ph-positive Chronic Myeloid Leukaemia

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Coulombel et al. [1] reported that Philadelphia (Ph) chromosome-negative progenitors, even when present at undetectable levels in the initial marrow specimen, may thus become demonstrable within two to four weeks of long-term marrow culture, whereas the Ph-positive population rapidly declines. Dubé et al. [2] observed that in some CML patients Ph-negative mitoses can be found in blood progenitors whereas all metaphases from marrow progenitors were abnormal. On the other hand, it is well documented that CML primitive progenitors respond to colony stimulating factors as the granulocyte/macrophage colony stimulating factor (GM-CSF) or the granulocyte colony stimulating factor (G-CSF) [3].

On the basis of these findings, we examined the PHA-LCM-stimulated whole blood liquid suspension culture of a patient with Ph-positive CML for leukaemic cell differentiation and for chromosome content and karyotypes.

I. N. (born 13. 06. 1978) was admitted to our Pediatric Department as an 8-year-old boy for respiratory tract infection associated with hepatosplenomegaly and anaemia. The marrow aspiration showed hypercellularity and contained 7% myeloblasts, 17% promyelocytes, 5% myelocytes, 3% metamyelocytes, 15% bands, 14% segmented neutrophils, 11% eosinophils, 6% basophils, 17% lymphocytes, 2% reticulum cells, and 3% normoblasts. The peripherial blood WBC was $24 \times 10^9/l$, with GAPA score of 26 (very low). The patient was treated with busulphan, hydroxyurea, prednisolone, and vincristine. His spleen was irradiated, but remission could not be achieved. The patient currently receives supportive treatment 6 months after diagnosis.

For cytological examination, a cell suspension from the 6-day culture was stained with May-Grünwald-Giemsa. Preparation of phytohemagglutinin-stimulated leukocyte-conditioned medium (PHA-LCM) was made as described by Park et al. [4]. Chromosome examination was performed during the supportive treat-

Table 1

Comparison of the cytological examination data

Leukocyte differential count %	Peripheral blood	6 day culture
Atypical blasts	39	34
Myeloblasts	6	11
Myelocytes	11	—
Granulocytes	—	14
Basophils	—	4
Eosinophils	2	—
Lymphocytes	40	3
Normoblasts	—	9
Monocytes	2	—
Macrophages	—	25

ment. For routine cytogenetic analysis, a 24 h unstimulated whole blood culture was investigated [5]. The liquid suspension culture method was performed as follows: 2 ml of heparinized whole blood from the patient was cultured in 10 ml of TC 199 (Difco) medium, supplemented with 10% fetal calf serum, 10% PHA-LCM, penicillin and streptomycin. The cells were cultured at 37 °C for 6 days in an Erlenmeyer flask closed with a sterile rubber stopper. On the 4th day of culturing, half of the medium was changed for fresh TC 199 medium supplemented with 10% fetal calf serum. GTG-banded chromosome preparations were made by the method of Autio & Schröder [6]. Karyotype analyses were carried out with the short version of ISCN [7]. The cytological examination (Table 1) showed that immature blast cells with both progenitor and mature cells of erythroid and granulocyte/macrophage lineage were present in the PHA-LCM-stimulated 6-day culture of the patient's blood, in contrast to his peripheral blood, in which the WBC was 14×10^9/l, the leukocyte differential count included mainly atypical blasts, lymphocytes, myelocytes, myeloblasts, eosinophils, and monocytes.

Chromosome examinations: twenty-eight (56%) of the fifty cells examined contained 45 chromosomes, while sixteen cells (32%) contained 46 chromosomes. In four mitoses (8%), the chromosome number was in the near-tetraploid region, while in two mitoses (4%) 44 chromosomes were found.

Table 2 shows that both numerical and structural aberrations were found in the twenty-five karyotypes examined. Three cytogenetically distinct clones were present: cells with the normal 46,XY karyotypes (in 6 cases), cells with the characteristic t(9; 22) (q34; q11) for the Ph-positive CML (in five karyotypes), and, cells with 45,XY,-7 karyotypes (in eight cases). The other aberrations were each observed in single mitoses. Only Ph-positive leukaemia cells were found in the

Table 2

Numerical and structural chromosome aberrations

Type of aberration	Number of cells with aberration
44, XY, −7, −16, t(9; 22) (q34; q11)	1
44, XY, −7, −17	1
45, XY, −7	8
45, XY, −7; t(9; 22) (q34; q11)	1
46, XY	6
46, XY, t(9; 22) (q34; q11)	5
86, XXYY, (4n±)−7, −12, −17, −18, −21, −22	1
88, XXYY, (4n±)−6, −7, −8, −12, t(9; 22) (q34; q11)	1
92, XXYY, t(9; 22) (q34; q11)	1

ten karyotypes examined from the unstimulated 24-h culture of the patient's blood prepared at the same time as the liquid culture was made.

The results presented here show that in the PHA-LCM-stimulated 6-day whole blood suspension culture, the leukocyte differential count changed toward the granulocyte/macrophage lineage compared to the peripheral blood of the patient. The presence of some near-tetraploid mitoses in the culture proves the ability of these cells to fuse with normal or leukaemia cells under these circumstances. Ph-positive leukaemic cells could be detected with this method. Cells with normal karyotypes can be primitive, Philadelphia chromosome-negative progenitors which were found in long-term marrow cultures [1]. The dominant clone was 46, XY, −7 in the culture. This chromosome loss occurs in only 3% of patients during the acute phase of CML [8]. Monosomy 7 was found in a patient with lymphoid transformation and a precursor B-cell phenotype [9], and in several cases documented by Mitelman [10]. The presence of cells with 45, XY,−7 karyotypes in the PHA-LCM stimulated 6 day whole blood culture of our patient indicated the lymphoid transformation of his CML, because the following differential leukocyte count was found in a later examination of his peripherial blood: (WBC : 16×10^9/l) 20% atypical blasts, 16% neutrophils, 2% eosinophils, and 62% lymphocytes.

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Abstracts

Cross bonding and stiffening of the red cell membrane. Th. M. Fischer (Institut für Physiologie, Medizinische Fakultät, Rheinisch-Westfälische Technische Hochschule, Aachen, F.R.G.). *Biochim. Biophys. Acta 985*, 218 (1989).

Cross bonding and stiffening of the human red cell membrane was studied using treatments with SH, amino, and carboxyl reagents, oxidizing and denaturing treatments and acidification. Membrane cross bonding was initiated when, after red cell treatment, opposite areas of the cytoplasmic face of the red cell membrane were brought into contact by cell shrinking. Membrane cross bonding was detected by light microscopy when this contact persisted upon swelling the cells in a hypotonic medium. Membrane stiffening was recorded as a decrease in elongation of red cells in the shear field of a viscous dextran solution. No correlation was found between membrane cross bonding and membrane stiffening. The results are explained by the existence of two modifications of spectrin, type I causing solely membrane stiffening, type II causing membrane cross bonding as well as membrane stiffening. The amino and carboxyl reagents caused only type I modification. The other treatments caused both types of modification although with varying proportions. The results support the previously suggested mechanism of membrane cross bonding which involves a rearrangement of spectrin similar to denaturation by heat or urea, a decrease in associations within the membrane skeletal network, and a lateral aggregation of membrane proteins. These changes are proposed to occur by the type II modification. The data further substantiate the membrane stiffening effect of inter- and intra-molecular cross linking of spectrin which is identified with the type I modification. Finally, hypotheses are presented concerning the mechanism of membrane stiffening due to type II modifications of spectrin.

Ilma Szász

Abnormalities in erythrocyte membrane band 3 in chronic myelogenous leukemia. M. Kundu, J. Basu, M. M. Rakshit and P. Chakrabarti (Department of Chemistry, Bose Institute and Hematology Unit, N.R.S. Medical College and Hospital, Calcutta, India). *Biochim. Biophys. Acta 985*, 97 (1989).

The anion transport activities of erythrocytes from patients with chronic myelogenous leukemia (CML) and normal donors were comparable. In CML erythrocytes, significant reduction in the number of ankyrin-binding sites, present in the cytoplasmic domain of band 3, may lead to partial loss of cytoskeletal anchorage to the bilayer and account for their increased Con-A agglutinability and heat-sensitivity.

Ilma Szász

A study to see whether phosphatidylserine, partial proteolysis and EGTA substitute for calmodulin during activation of the Ca^{2+}-ATPase from red cells membranes by ATP. J.P.F.C. Rossi and A.F. Rega (Instituto de

Quimica Fisicoquimica Biologicas, Facultad de Farmacia y Bioquimica, Junin Buenos Aires, Argentina). *Biochim. Biophys. Acta 996*, 153 (1989).

(1) The effects of treatments that mimic calmodulin in increasing the apparent affinity for Ca^{2+} were tested to see whether, like calmodulin, they also change the activation of the Ca^{2+}-ATPase from human red cell membranes by ATP at the low-affinity site. (2) Short incubations with either trypsin or acidic phospholipids such as phosphatidylserine increased the apparent affinity for ATP at the low-affinity site. (3) Under conditions in which it increased the apparent affinity of the Ca^{2+}-ATPase for Ca^{2+}, EGTA failed to change the activation by ATP. (4) As in calmodulin-bound Ca^{2+}-ATPase, compound 48/80 inhibited the activity of the enzyme in the presence of phosphatidylserine by lowering the apparent affinity for ATP at the low-affinity site, leaving the maximum velocity of the enzyme unaltered. (5) Compound 48/80 also inhibited the Ca^{2+}-ATPase after partial proteolysis, but in this case it lowered the maximum activity, leaving the apparent affinity of the enzyme for ATP at the low-affinity site unaltered. (6) Inhibition of the Ca^{2+}-ATPase by compound 48/80 in the absence of calmodulin suggests that the inhibitor can act directly on the enzyme.

Ágnes Enyedi

Morphological alterations in erythrocyte membranes induced by 9-amino-1,2,3,4-tetrahydroacridine and 9-aminoacridine. D.A. Palmieri, R. J. Jacob, and D. Allan Butterfield (Department of Chemistry and the Center of Membrane Sciences, University of Kentucky, Lexington, Kentucky, USA). *Biochem. Biophys. Res. Comm. 163*, 1351 (1989).

The effects of 9-amino-1,2,3,4-tetrahydroacridine (THA) and its fully aromatic analogue 9-aminoacridine (9-AA) on erythrocyte membrane morphology were investigated via scanning electron microscopy. The ghost population was categorized into four distinct classes and alterations in the relative amounts of these populations with drug addition were noted. The samples incubated in 9-AA had a significantly higher ($p < 0.001$) flat, two-dimensional cell population. This shift in morphology may be attributable to the unwinding of spectrin and the subsequent collapse of the membrane.

Ágnes Enyedi

Ca^{2+} transients and Mn^{2+} entry in human neutrophils induced by thapsigargin. B. Foder, O. Scharff and O. Thastrup (Department of Clinical Physiology and Nuclear Medicine, University Hospital, Copenhagen, Denmark). *Cell Calcium 10*, 477 (1989).

Human neutrophils, preloaded with the fluorescent probe, Fura-2, were exposed to Ca^{2+}-releasing agents. The monitored traces of fluorescence were transformed by computer to cytosolic Ca^{2+} concentration ($[Ca^{2+}]i$). Due to quenching of Fura-2, the addition of Mn^{2+} enabled us to compute the cytosolic concentration of total manganese ($[Mn])i$). The agents used were the novel Ca^{2+}-mobilizing agent, thapsigargin (Tg), the chemotactic peptide, formyl-methionyl-leucyl-phenylalanine (FMLP), and the divalent cation ionophore, A23187. The agents caused transient rises of $[Ca^{2+}]i$ and monotonous rises of $[Mn]i$, suggesting influx but no efflux of Mn^{2+}. The rise time of $[Ca^{2+}]i$ and the time constants and magnitude of the apparent Mn^{2+} influx were strongly dependent on the sequence of addition of the agonist and Ca^{2+}. Contrary to FMLP, Tg needed several minutes to exert its full effect on the rise of $[Ca^{2+}]i$ and on the influx of Mn^{2+}, the latter being dependent on two phases, activation and partial inactivation. Pretreatment with phorbol 12-myristate 13-acetate (PMA) inhibited the responses of Tg, FMLP and A23187. For comparison, human red blood cells were tested. Contrary to A23187, Tg did not induce Ca^{2+} uptake in ATP-depleted red cells but increased the Ca^{2+} pump flux in intact red cells by 10%. The experimental data and computer simulations on the granulocyte data suggest that time-dependent changes of both passive Ca^{2+} flux into the

cystol and Ca^{2+} flux of the plasma membrane pump are involved in the transient $[Ca^{2+}]i$ response.

Ilma Szász

Phosphoinositide hydrolysis in mitogen-stimulated human peripheral-blood T lymphocytes. S. King, G. Whitley, M. Salmon and A. Johnstone (Departments of Immunology and Pharmacology St. George's Hospital Medical School, Cranmer Terrace, London, SW17 ORE, UK). *Biochem. J. 262*, 747 (1989).

Both phytohaemagglutinin and antibodies to the CD3 molecule induced proliferation and phosphoinositide hydrolysis in human peripheral-blood T lymphocytes, but the magnitude of the inositol phosphate response was small and the rate of accumulation slow (significant increases in $Ins(1,4,5)P_3$ were observed only after 10 min). Hence this response differs from the well-characterized $Ins(1,4,5)P_3$ responses of many other systems. This slow response, its abrogation in Ca^{2+}-depleted medium, the slow and maintained increase in Ca^{2+} as measured by Quin-2, and the ability of the Ca^{2+} ionophore A23187 to stimulate $Ins(1,4,5)P_3$ accumulation all suggest that the increase in $Ins(1,4,5)P_3$ occurs, at least in part, as a result of receptor-mediated Ca^{2+} influx in mitogen-stimulated T lymphocytes.

Ágnes Enyedi

Structure, function and subcellular localization of a human platelet Ca^{2+}-ATPase. W. L. Dean (Department of Biochemistry, University of Louisville, Kentucky, USA). *Cell Calcium 10*, 289 (1989).

Human platelets contain a Ca^{2+}-ATPase in internal membranes that is essential for Ca^{2+} homeostasis. This Ca^{2+} pump has enzymatic properties quite similar to the sarcoplasmic reticulum (SR) Ca^{2+} pumps. Antibodies against the SR Ca^{2+} pump cross-react with the human platelet protein. However, the platelet Ca^{2+}-ATPase is approximately 10 kD larger than the SR pumps and exhibits a larger mRNA coding for the protein in a megakaryocyte tumor cell line. In addition, the platelet Ca^{2+}-pump may be localized in specialized internal membrane structures that function in Ca^{2+} uptake and release. These results suggest that the platelet Ca^{2+}-ATPase may represent a new class of internal membrane Ca^{2+}-pumps.

B. Sarkadi

Calcium induces membrane translocation of 12-lipoxygenase in rat platelets. A. Baba, S. Sakuma, H. Okamoto, T. Inoue and H. Iwata (Department of Pharmacology, Faculty of Pharmaceutical Sciences, Osaka University, 1 – 6 Yamada-Oka, Suita, Osaka 565, Japan). *J. Biol. Chem. 264*, 15790 (1989).

Translocation of soluble 12-lipoxygenase to membranes was examined in rat platelets. Preincubation of platelet homogenates with $0.1 - 10\ \mu M\ Ca^{2+}$ resulted in an increase in 12-lipoxygenase activity of the particulate fraction with a concomitant decrease in that of the soluble fraction. Kinetic parameters of 12-lipoxygenase of the soluble and membrane fractions were not changes in the presence of $10\ \mu M\ Ca^{2+}$. Ca^{2+}-induced association of 12-lipoxygenase to the particulate fraction was dependent on the amounts of platelet-soluble and membrane fractions but not on the incubation temperature. 12-Lipoxygenase activity associated with the particulate fraction was completely dissociated by reducing the concentration of Ca^{2+} to 10 nM. Ca^{2+}-induced association of the enzyme also occurred in the boiled- and trypsin-treated membranes but was significantly reduced in the phospholipase A_2-treated membranes. Soluble 12-lipoxygenase also associated to liposomes in a Ca^{2+}-dependent manner. Pretreatment of platelets with thrombin (0.5 – 5 units/ml) significantly caused a translocation of soluble 12-lipoxygenase to particulate fraction; in the time course study, the translocation was observed at the thrombin pretreatment of 1, 5, and 10 min. These results suggest that stimulation of platelets is followed by the translocation of soluble 12-lipoxygenase to membranes, which is mediated by physiological concentration of Ca^{2+}.

B. Sarkadi

Further characterization of the plasma membrane- and intracellular membrane-associated platelet Ca^{2+} transport systems. J. Enouf, R. Bredoux, N. Bourdeau, B. Sarkadi and S. Levy-Toledano (Unité Inserm, No. 150, Hopital Lariboisiere, Paris Cedex 10, France). *Biochem. J. 263*, 547 (1989).

Biochemical characterization of the Ca^{2+}-ATPases isolated from human platelet intracellular and plasma membranes is reported. A comparative study of the previously partly described plasma membrane Ca^{2+}-ATPase and the intracellular membrane Ca^{2+}-ATPase obtained simultaneously shows differences in the following parameters: (1) different kinetics of the two enzymes; (2) similar apparent affinity towards Ca^{2+} (10^{-7}M), though the intracellular membrane enzyme was inhibited at Ca^{2+} concentrations above 10^{-7}M; (3) different pH dependence with an activity maximum at pH 7 for the intracellular membrane Ca^{2+}-ATPase and no detectable pH maximum for the plasma membrane Ca^{2+}-ATPase; (4) a 10-fold difference in the ATP requirement of the two Ca^{2+}-ATPases; (5) different patterns of inhibition by vanadate. Finally, the possible regulation of the Ca^{2+}-ATPases was examined by studying the effect of chlorpromazine on the two Ca^{2+}-ATPase activities, with only the plasma membrane enzyme being inhibited. It is concluded that the two platelet Ca^{2+} transport systems show biochemical differences in spite of the previously shown similarity in the molecular masses of their Ca^{2+}-ATPases, thus conferring a definite specificity to the platelet system.

B. Sarkadi

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Changes in Nucleolar Morphology during Macrophage Development in Non-Hodgkin's Lymphoma: a Morphometric Study

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Nucleolar ultrastructure of macrophages developing in suspension culture from monocytes was studied in 20 patients with non-Hodgkin's lymphoma and 20 normal subjects. Morphometric measurements of nucleolar volume, surface area, volume fraction, surface-to-volume ratio and number of profiles per section were made over a 6 day period. Multivariate analysis of variance showed that culture time and subject group had significant effects: changes during macrophage development were less marked in the patient group, nucleoli were fewer, rounder and possibly smaller than normal. The results indicate disturbed macrophage development in non-Hodgkin's lymphoma and may be related to mononuclear phagocyte dysfunction.

Keywords: electron microscopy, macrophages, malignant lymphoma, monocytes, morphometry, nucleoli

Introduction

Previous studies in patients with malignant lymphoma demonstrated the presence of ultrastructural changes in both peripheral blood monocytes [1] and skin window macrophages [2, 3] which were thought to be the morphological counterparts of the mononuclear phagocyte dysfunction known to occur in these conditions [4]. Since nucleoli have a key role in the regulation of cell growth and protein synthesis and provide an essential link between nucleus and cytoplasm [5], it seemed important to investigate whether nucleolar morphology of mononuclear phagocytes was also affected in lymphoma patients. A preliminary ultrastructural study in blood monocytes suggested that it was, patients' cells having smaller nucleolar volumes and volume fractions within the nucleus, together with fewer nucleolar profiles per section, as compared to normal [6]. The question then arose whether similar changes would be present at the macrophage stage of development. In the present work, a method previously employed

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to investigate the development of monocytes into macrophages in normal subjects [7] has been used and morphometric measurements made on the nucleoli of mononuclear phagocytes of patients with non-Hodgkin's lymphoma (NHL), as well as on those of a group of healthy individuals.

Materials and Methods

Studies were made on blood samples from 20 patients with NHL and 20 normal subjects.

There were 11 males and 9 females in the NHL group, none of whom had received specific treatment; their mean age $\pm$ standard error (SEM) was 55 $\pm$ 3 years. According to the classification of Gerard-Marchant et al. [8], 7 patients had centroblastic diseases, 5 had peripheral T cell lymphoma, 4 were of the centroblastic-centrocytic follicular type, 2 of the centrocytic type, 1 had lymphoblastic (Burkitt's) lymphoma and 1 had thymic lymphoma. The lymphoma was thought to be of B cell origin in 14 cases, of T cell origin in 5 and one (thymic lymphoma) was unclassifiable. All 4 disease stages and both A and B symptom categories [9] were represented. 17 patients had reduced cellular immunity (as assessed by the response to phytohaemagglutinin compared with age and sex-matched controls and by decreased numbers of T lymphocytes in peripheral blood using a sheep red cell rosette test [10]).

Ten males and 10 females were included in the normal group; their mean age $\pm$ SEM was 33 $\pm$ 2 years. All were in good health at the time of sampling and denied taking any medication.

Cell preparation and electron microscopy

Approximately 35 mls of venous blood was obtained from each subject and defibrinated under sterile conditions. As described previously, mononuclear cells were separated, washed, divided into aliquots and cultured in suspension with autologous serum [11]. One aliquot of cells was studied prior to culture (Day 0 specimen) while others were examined after 1, 2, 4 and 6 days in culture, respectively. Following assessment of viability by trypan blue exclusion, the cells were prepared for electron microscopy by standard methods [7, 12]. Ultrathin sections of consistent thickness (silver-gold) were examined in a Philips 400T electron microscope. Mononuclear phagocytes were recognised by their characteristic ultrastructural appearances [13]: the complex surface microvilli and pinocytic vesicles, and the presence of typical lysosomal granules were particularly useful features in identification, while nuclear profile configuration and chromatin pattern were less useful. Lymphocytes, also present, were easily distinguished.

Nucleated profiles of mononuclear phagocytes were selected in an identical, predetermined and random manner. Starting at the centre of the grid, all nucleated macrophages showing complete profiles were photographed sequentially as they

were encountered in the line of drive; 11 to 18 micrographs were obtained from each subject at each culture interval (i.e. approximately 1300 cells per group in total). A standard graticule was used to calculate magnifications; these ranged from ×7000 to ×14000. Nucleoli were identified by their electron microscope appearances, taking care to distinguish "nucleolus associated chromatin" [14, 15].

Morphometry

Morphometric analysis was carried out using principles and methods similar to those described previously and was based on the concept of a model cell: assuming that nuclei formed a monodispersed population with respect to size and were spherical [7, 16]. The micrographs were covered with transparent test grids. Point and intersect counting was carried out on nuclear and nucleolar profiles using grid lattices of 1 cm and 0.5 cm, respectively. The counting was performed without knowledge of the subject group. Profile areas, areal fractions and surface-to-volume ratios were calculated, appropriate compensation being made for non-equatorial sectioning of the nuclei [7, 16]. The following nucleolar measurements were then obtained: volume (V_n), surface area (S_n), volume fraction within the nucleus (${}^{V}V_n$), surface-to-volume ratio (S/V_n) and number of nucleolar profiles per section. Sampling adequacy was confirmed by the mean values for relative standard error (${}^{V}V_n$) and SEM/$\bar{x}$ (nucleolar profile numbers) ranging from 0.05 to 0.07 [17].

Statistical analysis

Multivariate analyses of variance (MANOVA) were carried out on the overall nucleolar measurements to examine the effect of culture interval, subject group, age and sex. Prior to the computations, the data were transformed to natural logarithm values in order to improve conformity to model assumptions of multivariate normality and homoscedasticity. As the mean ages of the subject groups differed greatly, age was treated as a covariate in the between groups comparison; whether such age differences could account for any of the findings was also examined. Univariate analyses of variance (ANOVA) were carried out on the separate nucleolar measurements to aid in the interpretation of the MANOVA results. The analyses were carried out on an Olivetti M24SP micro-computer using a SPSS/PC+ statistical package. Probabilities of $p < 0.05$ and $p \leq 0.01$ were chosen as the *a priori* levels of significance for MANOVA and ANOVA, respectively. The more stringent requirement for the latter was to take into account the 5 separate comparisons [7], an individual significance level (α) of 0.01 being required to give an overall significance level (S) of 0.05 ($S = 1 - (1 - \alpha)^5$). In NHL patients, subgroupings based on histology, B/T cell type, disease stage, symptom category and cellular immunity status were considered to be too small for valid statistical testing.

Table 1

Nucleolar measurements: summary of quantitative analysis

		Results at each period of culture ($\bar{x} \pm$ SEM)					ANOVA	
		Day 0	Day 1	Day 2	Day 4	Day 6	Effect of time (p)	Normal vs NHL (p)
Volume (fl)	Normal	1.24±0.10	1.69±0.19	1.53±0.23	4.21±1.01	4.28±0.91	0.00	0.03
	NHL	1.07±0.10	1.33±0.09	1.36±0.18	2.30±0.33	3.13±0.65	0.03	
Surface area (μm^2)	Normal	11.36±0.65	13.11±1.11	11.48±1.29	21.71±4.14	20.06±2.88	0.01	0.00
	NHL	10.21±0.71	11.04±0.69	10.14±1.07	12.63±1.43	15.32±2.22	0.38	
Volume fraction within nucleus (%)	Normal	1.42±0.12	1.82±0.17	1.57±0.21	2.97±0.44	2.93±0.32	0.00	0.35
	NHL	1.34±0.12	1.69±0.12	1.64±0.19	2.29±0.29	2.59±0.37	0.11	
Surface-to-volume ratio (μm^2 fl^{-1})	Normal	9.50±0.36	8.24±0.49	8.97±0.64	6.49±0.44	5.70±0.39	0.00	0.32
	NHL	10.28±0.55	8.40±0.31	8.28±0.35	6.73±0.64	6.80±0.92	0.00	
Number of profiles per section	Normal	1.02±0.05	0.94±0.06	0.81±0.06	0.86±0.08	0.80±0.04	0.05	0.01
	NHL	1.02±0.06	0.88±0.06	0.76±0.07	0.65±0.06	0.69±0.04	0.00	

Results

The results of the morphometric analysis are shown in Table 1. MANOVA showed that there were highly significant effects on the nucleolar measurements due to culture time ($p < 0.001$ for both patients and normals) and to subject group ($p = 0.003$). Age had no effect on the results in either group and the group effect was independent of the differences in age. No evidence was found of significant differences between males and females in either group.

The results of the ANOVA on the individual measurements in respect of the culture interval are given in Table 1 (Effect of time). Unlike the normal group, the increases in V_n, S_n and ${}^VV_n$ in the NHL patients were not significant. Using 'spheres of equivalent volume' (S_e) to assess nucleolar irregularity [7], the ratio S_n/S_e decreased during culture from 2.02 to 1.48 in the NHL group and from 2.04 to 1.57 in the normal subjects, indicating that the decrease of $(S/V)_n$ in both groups during the 6 days were due to the nucleoli becoming rounder as well as to the volume increases. The results of the ANOVA comparing the subject groups are also shown in Table 1 (Normal vs NHL), values for S_n and number of nucleolar profiles per section being significantly smaller in NHL.

Discussion

The results are interpreted as showing that while the nucleolar morphology changed over the period of culture in both groups, there were significant differences between the NHL patients and the normals, in particular nucleoli were fewer and rounder in the patient group. The volumes of the nucleoli were also consistently smaller, though p at 0.03 for the differences in V_n was only suggestive of significance, not meeting the reqired value for the ANOVA of $p \leq 0.01$.

The findings are in keeping with other reports of ultrastructural disturbances in mononuclear phagocytes in NHL in vivo [1–3, 6]. Compared with normal subjects, skin window macrophages in NHL patients had less mature surface appearances [2] and their monocytes had larger nuclei with more euchromatin and a relatively smaller heterochromatin-euchromatin interface, cell profile areas were larger with smaller surface-to-volume ratios [1]. In a morphometric study of nucleoli of uncultured blood monocytes, values for V_n, S_n, ${}^VV_n$ and number of profiles per section were 19–32% smaller than normal [6]. Further studies of skin window cells suggested premature activation of macrophages in lymphoma patients associated with a subsequent defect in phagocytic potential [3]. Such changes are unlikely to be specific to NHL as similar findings have been reported in patients with Hodgkin's disease [1–3, 6]. However, the present results are consistent with a disturbed pattern of transformation of monocytes into macrophages in NHL, and this is supported also by other morphological studies during culture. The cells of NHL patients appeared to grow less, show less elaboration of surface features and have reduced nuclear and mitochondrial

development, the latter affecting mitochondrial numbers rather than size [18]. The ultrastructural changes may be related to the mononuclear phagocyte dysfunction observed in other investigations. Phagocytosis [19, 20], antigen dependent cellular cytotoxicity [21], microbicidal activity [22–25], superoxide anion production [26] and glucose metabolism [27] have all been reported as either enhanced or decreased in patients with NHL, although normal functions have also been found [23, 28].

Knowledge of the spatial organisation within mononuclear phagocytes is important in relation to understanding their multiplicity of defensive and regulatory functions. Nucleoli are key organelles in cell metabolism and the present results indicate that morphometry offers a relatively simple method of providing objective data on changes in their morphology. The experimental system used here has several advantages. Culturing mononuclear phagocytes with autologous lymphocytes and serum allows each sample to act as its own control, particularly in respect of effects due to being in vitro; yet the system would readily permit the assessment of individual factors which may modify nucleolar development: these include the co-cultured lymphocytes, serum factors (eg interleukins) and changes intrinsic to the mononuclear phagocytes themselves. The findings would likely add to our basic understanding of NHL.

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The Effect of Monocytic Involvement of Hyperaldolasaemia in Patients with Acute Myelogenous Leukaemia

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Serum aldolase concentrations were determined in 78 patients with acute myelogenous leukaemia (AML). Mean serum aldolase concentration ($\pm$ SD) at the time of diagnosis was 28.3 U/L (+19.4). 71.8% of AML patients had increased serum aldolase concentrations. The highest hyperaldolasaemia were observed in patients with acute myelomonocytic leukaemia and acute monoblastic leukaemia. The monocytic involvement in AML patients was significantly related to serum aldolasaemia. Human aldolase gene has been located on chromosome 16, whose structural aberrations are frequent in AML patients with monocytic involvement, explaining the link between serum hyperaldolasaemia and the monocytic involvement in AML patients.

Keywords: erythropoiesis, fructosediphosphate aldolase, monoblastic leukaemia, myeloblastic leukaemia

Introduction

Mammalian fructose 1,6-diphosphate aldolase is a tetrameric glycolytic red cell enzyme with three subunit types: aldolase A is predominantly high in muscle, aldolase B is found exclusively in liver and aldolase C exists predominantly in brain and nervous tissue [1]. All patients with hepatic malignancies had increased serum levels of aldolase which is the predominant isoenzyme in fetal tissue, suggesting an oncofetal reversion [2]. Further on, all patients with chronic myeloid leukaemia (CML) had increased serum levels of aldolase, which was consistent with a fetal erythropoiesis and increased glycolytic rate [3]. In patients with acute leukaemia, the production of fetal haemoglobin has been reported [4]. The present study was undertaken to define the initial serum aldolase concentrations in patients with AML and to correlate them to the various subtypes of AML. The results showed that 71.8% of AML patients had hyperaldolasaemia, which was significantly related to the monocytic involvement of their blast cells, suggesting a malignant reversion to fetal erythropoiesis in AML patients, particularly in those with monocytic involvement.

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Materials and Methods

Patients

Seventy eight patients (34 females and 44 males) with AML were studied. Mean age was 49.8 years (range 20 – 83). The diagnosis was based on cell morphology and cytochemistry and organised according to the French – American – British (FAB) classification scheme [5, 6]. All AML patients had normal liver function tests at the time of diagnosis.

Measurement of serum aldolase

Serum aldolase concentrations were measured in blood samples obtained from each AML patient at the time of diagnosis as described previously [7]. Control serum aldolase concentrations were measured in 40 normal blood donors and varied from 5.2 U/l to 7.6 U/l (mean 6.5 ± 1.8). Hyperaldolasaemia was defined as serum aldolase concentration higher than 7.6 U/l, which was the upper limit of normal serum aldolase concentration in the controls.

Results

At the time of diagnosis, the mean serum aldolase concentrations for the various FAB subtypes of AML patients were significantly increased compared to the normal controls (Fig. 1). Fifty-six of the 78 AML patients (71.8 %) showed hyperaldolasaemia. The highest serum aldolase levels were observed in patients with acute monoblastic leukaemia (AMoL)M5 (Fig. 1). The results also showed a significant difference ($p < 0.001$) in serum hyperaldolasaemia between patients with AMoL(M5) and patients with other FAB subtypes of AML (Table 1). A similar significant difference ($p < 0.001$) in serum hyperaldolasaemia was

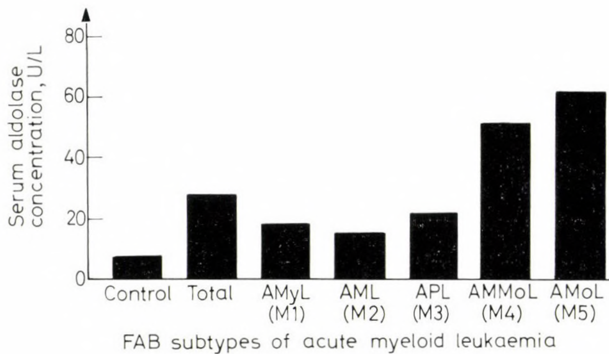

Fig. 1. Hyperaldolasaemia in acute myeloid leukaemia

Table 1

Effect of monocytic involvement on serum aldolase concentration in AML patients

FAB subtypes of AML	AMyL(M1) + AML(M2) + + APL(M3) + AMMoL(M4) (n = 65)	AMoL(M5) (n = 13)	p
Serum aldolase concentration (U/L)	21.4 ± 18.7*	69.0 ± 44.9	< 0.001

AMyL: acute myeloblastic leukaemia, AML: acute myeloblastic leukaemia with differentiation, APL: acute promyelocytic leukaemia, AMMoL: acute myelomonocytic leukaemia, AMoL: acute monoblastic leukaemia.

* Mean ± Standard deviation.

Table 2

Significance of monocytic involvement on serum hyperaldolasaemia in AML patients

FAB subtypes of AML	AMyL(M1) + AML(M2) + + APL(M3) (n = 54)	AMMoL(M4) + AMoL(M5) (n = 24)	p
Serum aldolase concentration (U/L)	17.3 ± 12.0*	56.3 ± 38.5	< 0.001

AMyL: acute myeloblastic leukaemia, AML: acute myeloblastic leukaemia with differentiation, APL: acute promyelocytic leukaemia, AMMoL: acute myelomonocytic leukaemia, AMoL: acute monoblastic leukaemia.

* Mean ± Standard deviation.

found between AML patients with monocytic involvement (acute myelomonocytic leukaemia and acute monoblastic leukaemia) versus those without monocytic involvement (acute promyelocytic leukaemia and acute myeloblastic leukaemias) (Table 2).

Discussion

The reappearance of fetal haemoglobin has been reported in human leukaemia [8]. In acute leukaemia, the reversion to a fetal form of erythropoiesis suggests the existence of a dyserythropoietic state in which the basic process is a disturbance of gene control mechanism leading to various metabolic alterations and post-synthetic modifications of proteins [9]. The major finding of the present study is that the serum hyperaldolasaemia which was manifested in 70% of AML patients, is significantly related to the monocytic involvement of their blast cells (Tables 1, 2). The highest serum aldolase level was observed in AML patients

with monocytic involvement (Fig. 1). Human aldolase gene has been recently located on chromosome 16 [10]. Structural aberrations of chromosome 16 has been very frequently reported in AML patients with monocytic involvement: acute myelomonocytic leukaemia (AMMoL) M4 [11, 12, 13] and AMoL M5 [14, 15, 16], which may explain the link between serum hyperaldolasaemia and the monocytic involvement in AML patients. Further studies to determine which of the three forms of aldolase (Aldolase –A, –B or –C) is the predominant cause of serum hyperaldolasaemia in AML patients with monocytic involvement may provide information on the types of fetal haemoglobin involved in human leukaemia.

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Phytohaemagglutinin-Induced Neutrophil Aggregation in Patients Affected by Chronic Myeloproliferative Diseases

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The aim of this work was to evaluate the phytohaemagglutinin-induced aggregation of circulating neutrophils isolated from 25 patients affected by chronic myeloproliferative syndromes (polycythaemia vera, chronic myeloid leukaemia in chronic phase, and essential thrombocythaemia).

The results showed a lesser aggregating capacity in chronic myeloid leukaemia (CML) and an opposite behaviour in polycythaemia vera and essential thrombocythaemia.

Patients with polycythaemia vera whose neutrophils showed a greater aggregating capacity were shown to have had various vascular complications.

Keywords: aggregation, CML, essential thrombocythaemia, phytohaemagglutinin, neutrophils, polycythaemia vera

Introduction

The aggregation of neutrophils represents an interesting event in acute inflammatory response. This neutrophilic function can be induced in vitro with various substances (zymosan activated serum, phytohaemagglutinin, calcium ionophore A23187, arachidonic acid, phorbol myristate acetate, thrombin, formyl-methionyl-leucyl-phenylalanine, leukotriene B4), and it leads to the production of lipo- and cyclo-oxygenase metabolites, which have a wide spectrum of activity in the modulation of immunity phenomena [1].

The aggregation of neutrophils has been, and still is, studied in various human pathological conditions, such as neutropenia, shock lung syndrome, angina pectoris and myocardial infarction, in which a possible pathogenetic role is attributed to the neutrophil aggregation itself (e.g. the vascular margination of neutrophils or the neutrophil-endothelium interaction [2–4].

To our best knowledge, the aggregation of neutrophils has not yet been studied in myeloproliferative diseases, in which various neutrophil functional defects have been noted [5].

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The aim of this study was to evaluate the aggregation of neutrophils induced by phytohaemagglutinin (PHA) in 25 patients affected by chronic myeloproliferative diseases. Lectins, in fact, are often used as stimuli for neutrophils: their action results in a number of alterations of cell function and structure [6, 7]. A study aimed at characterizing lectin-binding surface proteins has been published recently [7], this, however, did not involve PHA.

Materials and Methods

Subjects studied

Thirteen healthy volunteers were studied together with 25 patients affected by chronic myeloproliferative syndromes: 9 with polycythaemia vera (PV), 8 with chronic myeloid leukaemia (CML) in chronic phase and 8 with essential thrombocythaemia (ET) (Table 1).

The patients with ET were studied at the moment of diagnosis; those affected by CML were studied at least 8 weeks after any applied therapy; those affected by PV were studied at least 4 weeks after haemophlebotomy. One of these latter patients had been previously treated with pipobroman.

At the time of the study, none of the patients showed any symptoms attributable to vascular complications.

PV and ET were diagnosed according to the criteria of the American Polycythaemia Vera Study Group. The diagnosis of CML was based on clinical parameters, morphological study of bone marrow and the results of cytogenetic studies.

In the group of patients affected by PV we retrospectively evaluated any relationship between the results of neutrophil aggregation and the vascular symptoms (myocardial infarction, transient attacks of ischemia, venous thrombosis, microvascular thrombosis, haemorrhage) shown by any single subject throughout his/her illness.

Table 1

General characteristics of the patients studied

Diagnosis	n	Sex		Age (years)		Neutrophils	Platelets
		M	F	$\bar{x}$	range	($\times$ 10^9/l)	
PV*	9	5	4	65	49 – 86	2.8 – 13.5	200 – 861
ET**	8	4	4	63	40 – 72	3.0 – 20.0	700 – 1850
CML***	8	5	3	52	30 – 75	15.0 – 40.0	250 – 750

* Polycythemia vera.
** Essential thrombocythemia.
*** Chronic myeloid leukemia in chronic phase.

Neutrophil preparation

Neutrophils were obtained as described earlier [9] with modifications. Heparinized venous blood (10 units heparin/ml) was centrifuged at 700 rpm for 15 min and the supernatants were discarded in order to reduce platelet contamination. The blood was sedimented with dextran 1 : 1 (v/v) for 30 minutes at 37 °C. The leucocyte-enriched plasma was centrifuged at 160 *g* for 10 minutes. The cells were resuspended and washed twice at 160 *g* for 5 minutes in PBS containing neither calcium nor magnesium, sedimented on Lymphoprep and centrifuged at 400 *g* for 30 minutes. After the removal of the contaminating red blood cells by means of hypotonic lysis, the precipitated neutrophils were washed in PBS and re-suspended in the same solution containing bovine serum albumin 0.25%, calcium and magnesium at a concentration of 12×10^6 cells/ml. The cellular suspensions were stained by May–Grunwald–Giemsa and alpha-Naphtylacetate-esterase stains: lymphocyte and monocyte contamination was <1%. Platelet contamination was <1%. Samples from patients suffering from CML contained metamyelocytes <1–2%. The neutrophil viability, checked by means of trypan blue exclusion, was always greater than 99%.

Neutrophil aggregation

The aggregation was measured in a platelet aggregometer (Aggrecorder PA-3210, Menarini S. p. A., Florence, Italy), rotating continuously at 900 rpm. The aggregating agent was PHA (Difco), at final concentrations which varied fom 6 to 120 μg/ml. 250 μl of the neutrophil suspension was added to siliconized cuvettes. After heating the cells at 37 °C for 2 minutes, 50 μl of PHA was added. The registration system of the aggregometer was calibrated with PBS (containing bovine serum albumin 0.25%). Quantitation of granulocyte aggregation during

Table 2

Neutrophil aggregation in patients affected by chronic myeloproliferative syndromes

Diagnosis	PHA (μg/ml)		
	120	60	6
PV*	43.4 ± 4.08	37.6 ± 5.74 #	13.6 ± 3.52
ET**	41.0 ± 3.03	35.2 ± 3.76 ##	15.2 ± 3.76
CML***	37.6 ± 4.28 [a]	32.3 ± 4.21	12.2 ± 4.27
Controls	43.2 ± 2.99	29.2 ± 4.88	18.5 ± 7.60

Aggregation values are expressed in cm^2 (means ± SD).
* Polycythemia vera; ** Essential thrombocythemia; *** Chronic myeloid leukemia in chronic phase; # $p < 0.01$; ## p 0.02; [a] $p < 0.005$.
In no case was spontaneous aggregation noted.

the initial 5 min was made using a compensating polar planimeter (Mod 236, Salmoiraghi S. p. A., Milan, Italy) and the results were expressed in square centimeters. Aggregation was verified by phase contrast microscopy.

Statistics

The results were analyzed by Student's *t* test for unpaired data.

Results

PhA induced irreversible and dose-dependent aggregation.

The results are shown in Table 2. In 5 patients affected by PV and in 4 patients affected by ET, we observed an increase in neutrophil aggregation with PHA 60 μg/ml ($p < 0.01$ and < 0.02, respectively). In patients with PV this concentration of PHA seemed to be related to the vascular symptoms of each individual (Table 3).

Neutrophils isolated from patients with CML showed in 4 of 8 patients a reduction in aggregation with PHA 120 μg/ml ($p < 0.005$).

Table 3

Patients affected by polycthemia vera. Relationship between neutrophil aggregation obtained with PHA 60 μg/ml and vascular symptoms

Case	Aggregation	Thrombosis[1]	TIA[2]	MI[3]	Micr[4]	Haemorrhage
1	=	No	No	No	No	No
2	=	No	No	No	No	No
3	=	No	No	No	No	No
4	NT	No	YES	No	No	No
5	↑	No	YES	No	No	No
6	↑	No	No	No	YES	No
7	↑	YES	YES	No	No	No
8	↑	No	No	YES	No	No
9	↑	No	No	No	No	YES

NT: Not tested; = : Normal aggregating function; ↑: Increased aggregating function; [1]: Venous thrombosis; [2]: Transient attacks of ischemia; [3]: Myocardial infarction; [4]: Microvascular thrombosis.

Discussion

PHA-induced neutrophil aggregation is an irreversible phenomenon and the aggregation curves vary in morphology and values of aggregation according to the final concentration of PHA. In a preliminary paper [10] we observed that PHA-induced aggregation showed an evident dependence upon the transmembrane flow of calcium ions and calmodulin function; a limited dependence upon microtubular function; a response to the effect of cytochalasin B which varied according to the final concentration of this drug: an increase in aggregation at concentrations between 2 and 5 μg/ml, and a reduction in aggregation at the concentration of 10 μg/ml. Those results were in agreement with those reported by other authors who had used different aggregating agents [1, 11, 12].

Our results show that in some patients suffering from chronic myeloproliferative syndromes there are some anomalies in granulocyte aggregation. The aggregation behaviour seems to differ according to the disease in question: in the case of CML there seem to be above all a lesser aggregative capacity, while in the case of PV and ET the defect seems to be the opposite. Because of the relatively low number of cases studied, it is not possible to affirm that the 3 different diseases present typical profiles with regard to neutrophil aggregation. However, the different neutrophil aggregation behaviours shown could be a consequence of structural and/or functional anomalies related to the derivation of a part of the circulating neutrophils from one or more neoplastic clones.

Former studies have shown various anomalies in neutrophil functions and ultrastructure in CML patients [13–15]. More recently it has been shown that neutrophils both from subjects of acute and chronic myeloproliferative diseases can present various functional anomalies, which seem to be associated with a worse prognosis [5]. All these abnormalities could be compatible, in some patients, with aggregation anomalies in vitro.

Cooper et al. [16] have noted that neutrophils from patients affected by PV present a metabolic state which is higher both in the resting and in the phagocytic phase: an increase in the ingestion of latex particles, in the clumping during the phagocytic process and in the NBT reduction test.

Furthermore, to our best knowledge, no data are available on neutrophil functions in ET.

The tendency towards a higher aggregative capacity in PV may play a role in the pathogenesis of thrombo-embolic phenomena, particularly frequent in this myeloproliferative syndrome [17]. In fact, even if the cases studied are few in number, the 5 patients affected by PV, whose neutrophils have shown a greater aggregating capacity, were shown to have suffered from various vascular symptoms.

Venous thrombi partly consist of neutrophils, and it is possible that granulocyte-endothelium interaction and the phenomenon of intravascular neutrophil aggregation represent stages in the formative process of the thrombus. Recent in vitro studies support this hypothesis [18].

In conclusion, the study of neutrophil aggregometry could represent an interesting field of research in human pathology. Further studies are needed in order to confirm the results of this paper and determine the mechanisms responsible for the modifications in neutrophil aggregation in chronic myeloproliferative syndromes.

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Complement-Mediated Immune Complex Solubilization and Precipitation Inhibition in Sera of Patients with Non-Hodgkin's Lymphoma

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The complement functions of 42 patients with non-Hodgkin's lymphoma have been examined. The patients were divided into groups according to the severity of their disease: 1st — patients with high-grade lymphomas, 2nd — with low-grade lymphomas and 3rd — with chronic lymphocytic leukaemia. The adopted methods were the measurements of complement-mediated immune complex solubilizing capacity (CMSC) and the complement-mediated immune complex precipitation inhibition capacity (IPIC). The CMSC and IPIC values were examined parallel with CH50, C3 complement levels and with levels of circulating immune complexes (CIC) in the sera of patients. The results indicated that the acquired deficiency of complement functions could be established by CMSC and IPIC measurements in the sera of patients with high-grade lymphomas. These defects were found to be milder in the group with low-grade lymphomas, and were not detectable in CLL. The changes of CH50 levels were found to be similar to that of IPIC values and the decrease in C3 levels was detectable in high-grade and low-grade lymphomas too. Elevated CIC levels were found in those cases in which both CMSC and IPIC were decreased.

Keywords: non-Hodgkin's lymphoma, acquired complement deficiency, complement-mediated immune complex solubilization, complement-mediated immune complex precipitation inhibition

Introduction

Patients with malignant lymphomas are at an increased risk of infections. There are many factors predisposing to infection in this patient population: specific deficiencies in host defense mechanisms due to certain malignant processes and deficiencies in host resistance secondary to conventional chemotherapy. In the sera of these patients we frequently found elevated levels of circulating immune complexes (CIC). There are various clinical symptoms based on immune complex pathomechanisms observed in these patients, for example immunhaemolytic anaemia or vasculitis. The acquired defects in the complement system are an important aspect of these two abnormalities occurring in patients with lymphomas.

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Deficiency of complement-related opsonic activity or defects in the other protective complement functions may contribute to an increased incidence of bacterial infections and on the other hand cause an imbalance in the metabolism of immune complexes. The role of complement deficiencies in increased risk bacterial infections has been discussed by other authors [1, 2]. It is well-known that there are direct correlations between the impaired complement functions and the increasing levels of pathogenic immune complexes. Details of the mechanisms of this phenomenon have become clearer [3, 4, 5].

In 1975, Miller and Nussenzweig showed that complement components can solubilize preformed immune precipitates in vitro [6]. In the following years the complement-mediated immune complex solubilizing capacity (CMSC) was characterised as a function of the alternative complement pathway. The classical pathway is able to enhance this reaction but cannot promote an effective solubilization by itself [7]. After a few years Schifferly, Naama and their co-workers, studying the in vitro formation of immune complexes in normal sera, identified a new complement function: the complement-mediated immune complex precipitation inhibition capacity (IPIC) [8, 9, 10]. This function depends on the activity of the classical complement pathway. Both processes – the solubilization and the precipitation inhibition – result in the formation of soluble immune complexes with modified structures which form a covalent bond with complement fragments, similar to the complexes in vivo [5, 11].

In this study we adopted the methods, based on two phenomena, to examine the integrity of the complement system of our patients with non-Hodgkin's lymphoma. The CMSC and IPIC values were examined parallel with CH50, C3 complement levels and CIC levels in sera of patients. These patients were divided into groups according to the severity of their disease.

Materials and Methods

Patients and controls

Serum samples were obtained from 42 patients with non-Hodgkin's lymphoma and from 30 healthy subjects. The patients were divided into groups according to their histological diagnosis based on the Kiel Lymph Node Registry [12]. The three groups were: 14 patients suffering from high-grade, 14 others from low-grade lymphomas and a third group of 14 patients with chronic lymphocytic leukaemia (CLL). The CLL group was separated from other low-grade lymphomas. The patients were in complete or incomplete remission. They were not given cytostatic agents for more than one month before blood was taken and they had no bacterial or viral infections.

Serum samples

The blood was allowed to clot at room-temperature for one hour and then at +4 °C for two hours. Thereafter, the serum was separated by centrifugation

and divided into aliquots for the different tests. The serum samples for CMSC and IPIC were used freshly immediately after the separation.

Measurement of CMSC

The antigen components of the artificially produced complexes were radiolabelled. Bovin serum albumin (BSA) was labelled with ^{125}I by the chloramine-T method [13]. Cleaned rabbit IgG produced against BSA (Philaxia) was used for preparing the complex. At weighing the labelled BSA with anti-BSA, an approximately fourfold antibody excess was applied. The reaction mixture was incubated at 37 °C for two hours and then at 4 °C for 12 hours. The precipitate was separated by centrifugation at 1600 g for 15 minutes and was washed six times in PBS buffer (physiological NaCl solution buffered with phosphate). The washed precipitate was dispersed by repeated sucking through a thin cannule. The volume of complexes was adjusted to produce 70–80% CMSC in freshly mixed healthy sera and background CMSC with buffer or heat-inactivated sera was less than 10–15%. This precipitate was stored in small aliquots at −20 °C until used. The measurement of CMSC in sera of patients and controls was performed as described previously [14]. Briefly: 400 μl of the 1 : 2 dilution of serum was prepared with PBS^{++} (containing 0.15 mmol/l Ca^{++} and Mg^{++} ions). It was added to 20 μl IC solution in duplicate. The mixture was incubated at 37 °C for two hours. The reaction was stopped by ice-cold PBS. Segregation of the non-soluble precipitate and supernatant was performed by centrifugation at 1600 g for 15 minutes in a cooled centrifuge. After the segregation of supernatant and pellet the radioactivity of each was measured. Calculation of the rate of solubilization was performed by the formula:

$$\% \text{ CMSC} = \frac{\text{activity of supernatant}}{\text{activity of supernatant + pellet}} \times 100$$

The CMSC value of the mixed healthy sera was always defined as 100% and the values of the samples were compared to this standard.

Measurement of IPIC

Five μl ^{125}I labelled BSA solutions were measured into the polystirol tubes (≈ 1.5 μg BSA). Thereafter 100 μl of 1 : 2 dilutions of serum samples were added to each tube. The sera were diluted with PBS^{++}. Afterwards 10 μl of anti-BSA solution (≈ 4 μg anti-BSA) was measured into each mixture. The tubes were incubated at 37 °C for one hour. The reaction was stopped by ice-cold PBS. Segregation of precipitated immune complexes from the supernatant which contained the small soluble complexes was performed by centrifugation at 1600 g for 15 minutes. After the separation of the supernatant and pellet, the radioactivity of each was counted. The concentrates of antigen and antibody solutions were adjusted to produce 80–85% IPIC values in mixed healthy sera and back-

ground IPIC with buffer alone was less than 10%. Calculation of IPIC was performed by the formula:

$$\% \text{ IPIC} = \frac{\text{activity of supernatant} - \text{C}}{(\text{activity of supernatant} + \text{pellet}) - \text{C}} \times 100$$

C = activity of supernatant of buffer control.

The IPIC value of mixed healthy sera was always defined as 100% and precipitation inhibition values of the serum samples were compared to this standard. Our method was an adoptation based on the method of Füst et al. (personal communication).

Measurement of CH50 and C3 complement levels

The total haemolytic activity of classical pathway, (CH50), was measured according to Mayer [15]. Concentration of C3 was determined by single radial immundiffusion, using monospecific antiserum.

Measurement of CIC levels

The levels of circulating immune complexes were measured by a complement test according to Johnson et al. [16].

Statistical analysis

The results were expressed as mean ± standard deviation (SD). The analysis of results was performed with Student's *t* test.

Results

In Table 1, the mean solubilization capacity of patients and the controls are shown. The CMSC values of samples from both low- and high-grade lymphomas were found to be significantly lower than that of healthy controls. In Table 1, the number of the samples which were decreased in each group are shown.

The precipitation inhibition values are summarized in Table 2. In the group of patients suffering from high-grade lymphomas, more than half of the serum samples showed decreased IPIC values. In the low-grade group only five samples out of 14 were found to be decreased. The mean of IPIC showed a significant decrease in the group of high-grade lymphomas.

The values of the total haemolytic activity of the classical complement pathway (CH50) are shown in Table 3. The mean of patients with high-grade lymphomas was significantly lower than that of controls. The eight patients with

Table 1

The complement-mediated immune commplex solubilization capacity (CMSC) in sera of patients

Patients	Decreased +	CMSC %	
High-grade lymphomas	6/14	77.50 ± 16.72	$p < 0.001$
Low-grade lymphomas	4/14	85.50 ± 14.99	$p < 0.05$
CLL	0/14	100.85 ± 14.90	n. s.
Controls	0/30	95.13 ± 8.74	

n. s. = not significant
+ = number of levels below control's mean − 2 SD

Table 2

The precipitation inhibition capacity (IPIC) in sera of patients

Patients	Decreased +	IPIC %	
High-grade lymphomas	8/14	85.64 ± 12.23	$p < 0.001$
Low-grade lymphomas	5/14	93.62 ± 9.16	$p < 0.05$
CLL	0/14	100.92 ± 4.82	n. s.
Controls	3/30	99.00 ± 3.81	

n. s. = not significant
+ = number of levels below control's mean − 2 SD

Table 3

The total haemolytic activity of the classical complement pathway (CH50) in sera of patients

Patients	Decreased (<49 CH50/ml)	CH50/ml	
High-grade lymphomas (n = 14)	8/14	56.50 ± 21.89	$p < 0.001$
Low-grade lymphomas (n = 14)	2/14	71.58 ± 16.60	n. s.
CLL (n = 14)	0/14	85.01 ± 13.98	n. s.
Control (n = 30)	2/30	79.37 ± 14.92	

n. s. = not significant

high-grade lymphoma and three patients with low-grade lymphoma had decreased values.

The mean values of the C3 complement levels are shown in Table 4. The mean values of high-grade and low-grade lymphomas were found to be significantly decreased, compared to the mean of controls. The number of values below 0.8 g/l were 6 out of 14 in the high-grade group. In the low-grade group, only 1 out of 14 was decreased. The mean of this group is significantly lower because the normal range of C3 levels is relatively wide.

In Table 5, the values of CIC are summarised. Six out of 14 were elevated in the group of high-grade lymphomas and four of 14 in the group of low-grade lymphomas. The mean values of these two groups were significantly elevated as compared to that of controls.

Table 4

The C3 complement levels in sera of patients

Patients	Decreased ≤ 0.80 g/l	C3 levels g/l	
High-grade lymphomas (n = 14)	6/14	0.63 ± 0.19	$p < 0.001$
Low-grade lymphomas (n = 14)	1/14	0.98 ± 0.26	$p < 0.01$
CLL (n = 14)	0/14	1.32 ± 0.14	n. s.
Control	0/14	1.29 ± 0.24	

n. s. = not significant

Table 5

The circulating immune complex level (CIC) in sera of patients

Patients	Elevated $> 15\%$	CIC %	
High-grade lymphomas (n = 14)	6/14	26.23 ± 19.14	$p < 0.001$
Low-grade lymphomas (n = 14)	4/14	14.25 ± 7.72	$p < 0.01$
CLL (n = 14)	0/14	3.43 ± 2.48	n. s.
Control	0/14	2.90 ± 3.80	

n. s. = not significant

Discussion

The prognosis of patients suffering from malignant lymphomas are relatively favourable in our days as a result of intensive therapeutic modalities. Successful therapy may result in remission, longer survival or sometimes recovery. The host resistance, which is impaired in these patients, permits frequent life-threathening infections, both during the active period of the disease and remission. The acquired deficiency of the complement system is a more important aspect of predisposition for serious infection and, on the other hand, causes disturbance in the immune complex metabolism. It is possible that failure of the complement-mediated immune complex formation results in more pathogenic complexes and may play a role in the development of immune complex diseases [4, 17, 21]. The syndromes based on immune complex mechanisms frequently appear in these patient groups. The acquired complement deficiency is well documented in CLL: defects in the early components of the classical pathway were found in some cases by other authors [18].

Increase in the circulating immune complex levels was frequent in lymphoid malignancy [19, 20]. The evaluation of this fact widely differs in literature. Some of the authors interpreted the elevated CIC levels as a prognostic factor [19, 20, 22], while others failed to observe a close correlation between the worse prognosis and a higher CIC level. It seems that the elevated CIC levels do not correlate directly with disease activity but mean first of all the deficiency of complement functions. These impaired functions may cause defects in actual host resistance, different syndromes based on the immune complex pathomechanisms and may result indirectly in a poorer prognosis by itself.

The measurement of solubilization and precipitation inhibition capacity are simple, reliable, reproducible and does not require expensive complement technology. These two methods were worked out earlier as diagnostic methods in the search for autoimmune diseases [10, 14, 17]. It was found that these assays appeared to be better methods for the early indication of disease activity than the measurement of CIC levels [8, 14, 17].

Our recent results indicate that the acquired deficiency of complement functions can be established by CMSC and IPIC measurement in the sera of patients with high-grade non-Hodgkin's lymphomas even in time of remission. These defects were found to be milder in the group of low-grade lymphomas and were not detectable in the group of patients with CLL. The defective complement functions or decreased levels of early complement components were reported by the authors on close to half of the patients with CLL [18, 23, 24], but our results did not show the same frequency as theirs. The difference appeared because our CLL group was selected under strict criteria: they were in the 0 and 1 Rye stage. We have not yet measured the levels of those components, but in the functional tests like CH50 or IPIC the smaller decreases of the components are moderately balanced. Our results indicate that these defects may be more frequent in the types of lymphoma which show higher malignancy. The change

of CH50 levels was found to be similar to that of IPIC values, and the decrease in C3 levels was detectable in the high-grade and low grade lymphomas, too. Elevated CIC levels were observable in those cases in which both CMSC and IPIC were decreased. The causes of decreased solubilization and precipitation inhibition capacity are more complicated in lymphomas than they are in autoimmune diseases. The depressed protein synthesis and toxic effects of the tumor or the treatment are associated with complement consumption which can appear during the immune complex formation and the elimination of infective agents.

These recent examinations were carried out in the remission period of the disease. These results would serve as a basis for our following investigations in time of disease activity and for measurement of therapeutic effect on these parameters. Our conclusion is that the solubilization and precipitation inhibition capacity can be used as prognostic factors during the course of malignant lymphomas. There is another important aspect of determining the impaired complement function: it could be substituted by using fresh, active complement containing plasma at the time of infection and to prevent serious acute inflammations due to immune complexes.

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Haemolytic Disease of Two Newborns in a Rhesus Anti-e Alloimmunized Woman. Review of Literature

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Haemolytic disease of the newborn (HDN) due to Rhesus anti-e alone is rarely observed in the Caucasian population. We report here a case of an R_2R_2 mother who had never been transfused and whose two children had a mild HDN without transfusion or blood exchange transfusion (BE) after delivery.

Keywords: anti-e, pregnancy, haemolytic disease, newborn infants

Introduction

In 1944, McIvor and Lucia [1] described the first case of HDN due to Rhesus anti-e antibody. Since then, few cases of HDN with anti-e antibody have been reported (Table 1). HDN with anti-e antibody is rarely encountered. In fact,

Table 1

Cases of anti-e alloimmunization alone observed in pregnant women with haemolytic disease of the newborn

Authors	Sex	Assumed genotype	DAT	BE	Clinical outcome
Pettenkofer et al. (1953)	female	CDE/cde	+	No	good
Von Bresgen et al. (1956)	male	cDE/cde	+	Yes	death
Pepperel et al. (1977)	–	–	+	No	good
Hanzlick and Senhauser (1979)	male	CDe/cDE	+	No	good
Chapman and Waters (1981)	female	cDE/cde	+	No	good

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in their study of 72,138 mothers, Pepperell et al. [2] found only one pregnancy with anti-e alloimmunized patient and positive direct anti-human globulin test (DAT) on cord blood of the baby. One case of mild HDN was observed by Hanzlick and Senhauser [3] and recently by Chapman and Waters [4].

We report here two cases of HDN due to anti-e in a family where the mother had not been transfused previously.

Materials and Methods

Standard methods (enzyme treated red blood cells (papain), anti-human globulin test in low ionic strength saline and polybrene autoanalyzer), according to AABB methods [5] were used for detection and titration of the alloantibodies in maternal serum and cord blood. Haemoglobin, bilirubin and the Liley index were evaluated by spectrophotometry.

Case Report

A caucasian woman, Mrs. ROD . . . born in 1946 in Poland whose blood group was AB Rhesus positive (cDE/cDE) has never been transfused. The husband's blood group was 0 Rhesus positive (CDe/CDe). In 1967, she became pregnant and a boy was born at 38 weeks of pregnancy. Seven years later, after a second pregnancy without complications, a girl was born at 37 weeks of pregnancy. No HDN was observed in these two newborn infants. Furthermore, no transfusion or BE was required after delivery. In 1977, during the third pregnancy, she had a

Table 2

Detection of anti-e with three methods and evolution of titre during the fifth pregnancy

Weeks of pregnancy	Methods		
	Papain treated red blood cells	Anti-human globulin test	Polybrene autoanalyzer
10	+(16)	–	+
17	+(32)	–	+
23	+(16)	+(16)	+
27	+(32)	+(16)	+
32	+(ND)	+(64)	+
35	+(128)	+(128)	+

( ) = titre; ND = not determined

Table 3

Evolution of bilirubin concentration in serum after delivery of the fourth child

Time after delivery (hr)	Concentration (μmol/l)
0	48
4	75
20	115
28	140
48	158
72	225
96	182

spontaneous abortion at 8 weeks of gestation. In 1978, during the fourth pregnancy, by the 27th week of gestation, an alloantibody anti-e was detected with the three methods, and its titer was 32 in anti-human globulin test. At 32 weeks of pregnancy, the anti-e alloantibody was again found with the three methods and its titre rose weakly to 64 in anti-human globulin test. Examination of the amniotic fluid showed that the Liley index values were 0.02 and 0.01 at 32 and 35 weeks of pregnancy, respectively. At 38 weeks of pregnancy, after spontaneous delivery, a girl was born and her blood group was A Rhesus positive (CDe/cDE. On cord blood, the DAT was positive(+), bilirubin and haemoglobin were 36 μmol/l and 165 g/l respectively. The outcome of this HDN was good and no BE or transfusion was needed. In 1981, during the fifth pregnancy, the anti-e was still detected in maternal serum. The variations of anti-e titre are shown in Table 2. The Liley index was determined three times during this pregnancy and their values were 0.022, 0.014 and 0.01 at 32, 35 and 38 weeks, respectively. In December 1981, a girl was born and her blood group was found to be B Rhesus positive (CDe/cDE). On cord blood, DAT was positive(+), bilirubin and haemoglobin were 48 μmol/l and 190 g/l respectively. The evolution of bilirubin concentration is reported in Table 3. No BE or transfusion was required.

Discussion

This report describes a case of an alloimmunized mother with anti-e antibody alone and whose two babies presented a positive DAT on cord blood and a mild HDN. The mother had never been transfused and the only immunizing stimulus was previous pregnancies. HDN due to anti-e antibody alone is rarely observed in the Caucasian population. This phenomenon can be explained partly by the high incidence of the Rhesus-e antigen in this population and partly by

the relatively low potency immunogenicity of this antigen [6]. In fact, Hanzlick and Senhauser [3] reported a case of a newborn infant with a haemolytic disease due to an anti-e antibody with good outcome. Similar data are given by Chapman and Waters [4] and Pepperell et al. [2]. Nevertheless, Bresgen et al. [7] have observed an HDN due to anti-e antibody with the death of the child despite BE. In the present case, the anti-e antibody titre rose slightly during the fourth pregnancy and markedly during the fifth pregnancy (Table 2). Despite this increase, the Liley index values did not suggest foetal damage. Further, no BE or transfusion was required for these two newborn infants after delivery. This observation agrees with the data of Hanzlick and Senhauser [3] who have observed a HDN in a first baby and probably in a second without transfusion or BE in an anti-e alloimmunized woman.

In conclusion, this report confirms that HDN due to anti-e antibody alone remains generally mild even after some pregnancies with antigenic stimulus and after a first case of HDN in the family.

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MAGYAR TUDOMÁNYOS AKADÉMIA KÖNYVTÁRA

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Defective CSA-Dependent Granulopoiesis in Patients with Chronic Drug-Induced Neutropenia

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Colony stimulating activity (CSA) and granulocyte-macrophage progenitor cells (GM-CFC) were assayed in the bone marrow and peripheral blood of 17 patients with drug-induced chronic neutropenia. Leukocyte-derived and monocyte/macrophage-derived CSA from the neutropenic patients was found to be singnificantly decreased compared to normal control. However, bone marrow and peripheral blood GM-CFC were within normal limits. These data suggest that in neutropenic patients monocyte/ macrophages exhibit most likely a qualitative defect in CSA production, which may account at least in part, for the impaired granulopoiesis observed in drug-induced neutropenia.

Keywords: GM-CFC, CSA, neutropenia, granulopoiesis

Introduction

Chronic neutropenia is a term used to describe a heterogeneous group of disorders with reduced numbers of circulating neutrophils for prolonged periods of time, without any evidence of an underlying disease known to be associated with granulocytopenia. Infantile genetic agranulocytosis, chronic idiopathic neutropenia, autoimmune neutropenia, chronic hypoplastic neutropenia and drug-induced chronic neutropenia, are included in this group of disorders [1–5]. Efforts to clarify the mechanism of granulocytopenia in these disorders have yielded very limited information so far.

In the present study we summarize our observations on some parameters of granulopoiesis in a group of patients with drug-induced chronic neutropenia.

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Materials and Methods

Seventeen neutropenic patients (4 males and 13 females) aged 15 to 68 years were included in this study (Table 1). The patients fullfilled the following criteria: 1) Circulating neutrophils $<1.700/\mu l$ of blood; 2) No concomittant monocytosis; 3) Bone marrow myeloid : erythroid ratio equal or $<3:1$; 4) Percentage of morphologically recognized proliferating granulocytic cells (proliferating pool) $<25\%$ of the total number of nucleated marrow cells; 5) Percentage of marrow non-proliferating granulocytic cells (maturation pool) $<35\%$; 6) Absence of splenomegaly; 7) No evidence of any underlying disease known to be associated with neutropenia; 8) No previous exposure to irradiation or use of cytotoxic drugs; 9) Negative leuko-agglutination test; 10) No evidence of familial or cyclic neutropenia, and 11) Absence of increased sensitivity to bacterial infections.

Table 1

Clinical data of the 17 neutropenic patients

Case #	Age/sex	Drugs received	Duration (months)
1	49, M	Dipyrone, aminopyrine	4
2	62, F	Ibuprofen	3
3	34, F	Indomethacine, sulindac, meprobamate, diazepam	6
4	50, F	Carbimazole, diazepam, dipyrone, insecticides	3
5	62, F	Indomethacine, phenylbutazone	4
6	50, F	Lorazepam, diazepam	10
7	23, F	Oxyphenbutazone	4
8	68, M	Phenylbutazone, dipyrone, ampicillin, insecticides	18
9	48, M	Ergoloid mesylates, dipyrone	6
10	47, F	Trimethoprim-sulfasoxazole, dipyrone	10
11	56, F	Ibuprofen, diazepam	10
12	50, F	Diazepam, dipyrone	2
13	54, F	Carbimazole	5
14	55, F	Diazepam	8
15	15, M	Carbamazepin	11
16	60, F	Sulindac	7
17	40, F	Diazepam, dipyrone	2

Table 2

Distribution of bone marrow granulocytic cells in neutropenic patients and normal controls

	Proliferating pool[1]	Maturation pool[2]	Mean Myeloid/ Erythroid ratio
	(%)		
Patients	19.2±1.2 (n = 17)	31.7±2.2 (n = 17)	1.93
Controls	17.0–28.0	35.0–50.0	3.00

[1] Blast cells, Promyelocytes and Myelocytes
[2] Metamyelocytes, Stab cells and PMNs

All patients had previously received one or more non-cytotoxic drugs and/or had been exposed to insecticides, as shown in Table 1. In most cases these compounds had been used for a period ranging between a few days and several months. The use of drugs and the contact with the insecticides had been discontinued at least two months before the study. Based on the above criteria and the previous history of the patients (Table 2), we classified their neutropenic state as drug-induced, although the causes of neutropenia might not have been necessarily uniform.

Preparations of Cell Suspensions

Unfractionated peripheral blood leukocytes (UF PBL) were obtained by allowing heparinized venous blood to settle at room temperature for 1–2 hours. The separated leukocyte-rich plasma was then removed and the leukocytes were used either for preparation of stimulating soft-agar underlayers (see below), or as target cells for colony formation.

Unfractionated bone marrow cell suspensions (UF BMC) were obtained as above. Following centrifugation, the buffy coat was removed and the marrow cells were suspended in supplemented MacCoy's 5A medium containing 5% fetal calf serum (FCS).

Suspensions of non-adherent light density cells (LDNA) were prepared from peripheral blood or bone marrow light density mononuclsar cells (LD) isolated by the Ficoll/Hypaque method. 5×10^6 peripheral blood or bone marrow LD cells were placed into Petri dishes (Falcon Plastics, Los Angeles Ca.) with 2 ml of supplemented MacCoy's 5A medium-5% FCS and were incubated for two hours in a fully humidified incubator of 37 °C and 7.5% CO_2 in the air. The non-adherent LD cells were collected by washing the plates twice, centrifuged, tested for viability and resuspended in medium. Approximately 95% of LDNA cells obtained by this procedure, were small lymphocytes.

Preparation of Monocyte/Macrophage Conditioned Medium

One ml of supplemented MacCoy's 5A medium containing 15% FCS [6] was added to each Petri dish, immediately after the removal of LDNA cells. Light density cells (LDAC) adhering to the bottom of the plates were then submitted to a further incubation for 5 days in the humidified incubator. Afterwards, the supernatant fluid, called monocyte/macrophage conditioned medium (MoCM) was collected, centrifuged, filtered and stored at -20 °C until used. The number of LDAC per plate was determined by the difference between LD cells initially plated and LDNA cells recovered after washing of the dishes. Approximately 98% of LDACs obtained by this method were esterase positive and were either monocytes or macrophages.

Assays for Colony Formation

All assays for colony formation by UF BMC, UF PBL or LDNA cells were performed in triplicate in 35×10 mm Petri dishes (Falcon) according to the method of Pike and Robinson [6]. 10^6 normal UF PBL in underlayers were used as source of exogenous colony-stimulating activity (CSA). After 10–12 days of incubation all clusters containing over 50 cells were counted as colonies. Cellular composition of picked-off colonies was determined after staining with 0.6% orcein in 60% acetic acid.

Assays for Colony-Stimulating Activity (CSA)

Leukocyte-derived CSA was assayed by incorporating 10^6 UF PBL in underlayers of 1 ml of 0.5% agar in supplemented MacCoy's 5A medium containing 15% FCS into Petri dishes. These underlayers were tested for CSA against 10^6 normal UF PBL suspended in 1 ml of 0.3% agar-medium overlayers. Colonies were counted after 10–12 days of incubation.

Monocyte/macrophage-derived CSA in conditioned medium was determined by the method of Pike and Robinson [6], slightly modified. 0.15 ml of MoCM were placed into Petri dishes and were tested for CSA against normal GM-CFC.

Results

Bone marrow granulocyte/monocyte colony forming cells (GM-CFC) of the neutropenic patients are shown in Table 3. In cultures stimulated with exogenous CSA, no statistically significant differences were observed between the neutropenic and the control group. In unstimulated cultures, colony formation was noted in 1 out of 7 patients and in 5 out of 9 normal subjects, when UF BMC were tested for autostimulation. These distributions were not statistically significant. Autostimulation was not observed in either group, when LDNA BMC were cultured in vitro.

Table 3

Bone marrow GM-CFC in neutropenic patients and normal controls

	GM-CFC/10^5 UF BMC[1]		GM-CFC/0.25 × 10^5 LDNA BMC[1]	
	Without exogenous CSA	With exogenous CSA[2]	Without exogenous CSA	With exogenous CSA[2]
Patients	0.2 ± 0.2 (n = 7)	35.5 ± 3.1 (n = 17)	0 (n = 8)	49.8 ± 4.6 (n = 9)
Controls	1.7 ± 0.7 (n = 9)	31.7 ± 4.3 (n = 11)	0 (n = 6)	44.3 ± 5.3 (n = 6)

[1] Values expressed as means ± 1 SEM.
[2] 10^6 UF PBL taken always from the same normal donor were used as source of exogenous CSA.

Table 4 shows the numbers of GM-CFC in patients' peripheral blood. In cultures stimulated with exogenous CSA, a statistically significant difference ($p < 0.05$) was found between GM-CFC values of the two groups, when UF PBL were tested. However, this difference was not observed when LDNA PBL were cultured in vitro, under the same experimental conditions. In unstimulated cultures, colony formation was noted in 2 out of 9 patients and in 5 out of 19 normal subjects when UF PBL were tested for autostimulation (data not shown). These distributionstare not statistically significant at the level of 5%.

Analysis of the type of colonies which were formed in vitro by patients' GM-CFC, did not reveal any statistically significant differences compared to those of the controls.

The capacity of patients' UF PBL to stimulate colony formation by normal GM-CFC was found to be significantly impaired compared to the control group

Table 4

Peripheral blood GM-CFC in neutropenic patients and normal controls

	GM-CFC/10^6 UF PBL[1]		GM-CFC/0.25 × 10^6 LDNA PBL[1]	
	Without exogenous CSA	With exogenous CSA[2]	Without exogenous CSA	With exogenous CSA[2]
Patients	0.2 ± 0.1 (n = 9)	34.2 ± 2.9 (n = 17)	0 (n = 9)	60.1 ± 5.6 (n = 11)
Controls	0.5 ± 0.2 (n = 19)	27.9 ± 1.6 (n = 47)	0 (n = 21)	49.7 ± 4.5 (n = 21)

[1] Values expressed as means ± 1 SEM.
[2] 10^6 UF obtained always from the same normal donor, were used as source of exogenous CSA.

Table 5

CSA capacity of UF PBL in neutropenic patients and normal controls

	Colonies per 10^6 UF PBL[1]	t-value	Degree of Significance
Patients	21.1 ± 2.5 (n = 15)	2.73	$p < 0.01$
Controls	28.4 ± 1.4 (n = 31)	—	—

[1] 10^6 normal UF PBL were used as target cells. Values are expressed as means $\pm$ 1 SE.

Table 6

Monocyte/macrophage-derived CSA in conditioned medium in neutropenic patients and normal controls

	Number of LD cells plated[1]	Mean number of LDAC ($\times 10^6$)	Colonies[2]	
			Per 0.15 ml of CM	Per 10^6 LDAC in 1 ml CM
Patients	5×10^6	1.4 ± 0.1 (n = 11)	15.6 ± 2.9 (n = 13)	59.7 ± 12.0[3] (n = 11)
Controls	5×10^6	1.6 ± 0.3 (n = 6)	28.0 ± 3.1 (n = 7)	116.8 ± 11.3[3] (n = 6)

[1] In each case, 3 to 5 Petri dishes were used.

[2] 10^6 normal LD PBL obtained always from the same donor, were used as target cells. The values are expressed as means $\pm$ 1 SE.

[3] Statistically significant difference between patients' and controls' values at the level of 1%.

($p < 0.01$) as shown in Table 5. Additionally, a statistically significant decrease ($p < 0.01$) in patients' LDAC-derived CSA in conditioned medium was documented, as shown in Table 6. The latter finding is not due to a subnormal number of adherent cells of the patient group, since the mean numbers of LDAC that remained at the bottom of Petri dishes, after the removal of LDNA cells, did not differ significantly from the control.

Finally, attempts to establish any correlation between patients' GM-CFC or CSA to some of the hematologic parameters, failed to demonstrate a statistically significant dependence of leukocyte-derived CSA from the number of circulating monocytes ($r = 0.165$) or the number of leukocytes ($r = 0.025$). Similarly, no statistical significant relationship was found between bone marrow GM-CFC and the marrow granulocyte proliferating pool ($r = 0.48$) or the number of stab cells and mature neutrophils ($r = -0.06$).

Discussion

It is well established that granulopoiesis involves interactions between non-proliferating cells producing regulatory factors and proliferating cells which respond to these factors [7]. In humans, monocytes/macrophages [6, 8 – 11], lymphocytes [10, 12], endothelial cells [13] and some other stromal cells [14] have already been recognised as the most important microenvironmental components producing stimulatory factors, i.e. CSA. Granulocyte/macrophage progenitor cells (GM-CFC) and morphologically identifiable granulocytic precursor cells are capable of responding to CSA and enhance their proliferation and maturation rate [14 – 15]. The system is under the control of several inhibitors of granulopoiesis, such as prostaglandins [16 – 17], interferons [18 – 19] or lactoferrins [20].

The data presented here indicate that CSA production by monocyte/macrophages is defective in patients with drug-induced chronic neutropenia. Both UF PBL in underlayers and monocyte/macrophage-derived CSA in conditioned medium from the neutropenic patients, were less capable of stimulating colony formation on normal GM-CFC compared to the normal control group. These findings are not due to quantitative differences in monocytes in the underlayers, since the absolute number of patients' monocytes in 10^6 UF PBL seeded in underlayers was equal to or higher than that of the control underlayers. Additionally, the number of monocyte/macrophages (LDAC) was adjusted when CSA in conditioned medium was studied (Table 6). It is conceivable that the low CSA values in our patients should be related to some qualitative abnormality of CSA-producing cells. Whether this abnormality is a result of a direct effect of the drug on the intracellular CSA-producing system of monocyte/macrophages or related to some alterations on CSA production directly [21] or indirectly [17, 22 – 23] remains unclear. It is noteworthy that the levels of serum CSA and of several inhibitors of granulopoiesis in the group of neutropenic patients were within normal limits (data not shown). Decreased CSA values in leukocyte-derived and in LDAC-derived MoCM were observed in 2 out of 6 patients with various types of chronic neutropenia [24]. Decreased CSA concentrations in bone marrow LDAC-derived MoCM have been also documented in 41 patients with chronic idiopatic neutropenia [25].

Colony-forming capacity of patients' BMC and PBL was within normal limits. Randomly selected colonies from patients' cultures showed a normal pattern of differentiation of GM-CFC to mature granulocytic forms. These observations indicate that neutropenia in patients with drug-induced chronic neutropenia is not associated to an inherent abnormality of GM-CFC for proliferation or differentiation. Normal levels of GM-CFC in peripheral blood and bone marrow have been found in 9 patients with chronic neutropenia due to non-cytotoxic drugs as in our group [5]. Furthermore, in cases of chronic idiopathic neutropenia, marrow and peripheral blood GM-CFC were either normal or slightly increased [25 – 26].

Although the mechanism of overall regulation of granulopoiesis has not been elucidated entirely yet, it is conceivable that patients with macrophages defective for production of CSA in sufficient amounts, might potentially become neutropenic [24]. It has been shown that little triggering is enough to stimulate stem cell proliferation and differentiation [27], while proliferation, differentiation and maturation of morphologically recognizable granulocytic precursor cells requires higher concentrations of the inducer stimulus [15]. If this is the case, it is possible that in low concentrations of CSA, the bone marrow maturation pool of granulocytic series would be decreased e.g. 'maturation arrest'. In our patients with low CSA values, the maturation pool was below the lower level of the normal range (Table 2), while the proliferating pool and GM-CFC values were within normal limits (Tables 2 and 3). Thus, one could postulate that in patients with drug-induced chronic neutropenia, granulopoiesis is regulated at a lower level compared to normal, most probably as a result of inadequate amounts of functional CSA liberated by macrophages into the hematopoietic microenvironment.

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Comparison of Some Lymphocyte Markers in B-cell Chronic Lymphocytic Leukaemia and Systemic Lupus Erythematosus (The B Lymphocyte Subset)

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Spontaneous mouse red-cell rosette formation (M-rosette), Leu 1 (CD 5) monoclonal antibody binding, ecto-nucleotidase enzyme reactions and mitogen responses of peripheral blood lymphocytes in 10 patients with B-cell chronic lymphocytic leukaemia (CLL), in 8 with systemic lupus erythematosus (SLE) and in 10 controls were studied.

The numbers of Leu 1 (CD 5) positive B lymphocytes in CLL and SLE were higher than in controls. These are called "autoregulatory" B lymphocytes, which are thought to be independent of T-cell regulatory effects. A significant increase of mouse rosette forming cells (MRFCs) was found in CLL, while in SLE and in controls their number was low.

Compared to the B lymphocytes of SLE patients and controls, those of CLL patients showed diminished responses to pokeweed mitogen stimulation parallel to their decreased level of 5'-nucleotidase.

Correlations between these markers and the features of isolated M-rosette positive CLL B lymphocytes are discussed.

Keywords: B-cell chronic lymphocytic leukaemia, systemic lupus erythematosus, M-rosette, Leu 1 (CD 5), 5'-nucleotidase, pokeweed mitogen responses, autoregulatory B lymphocytes

Introduction

Cells forming mouse red-cell rosettes spontaneously (MRFCs) represent a distinct population of B lymphocytes in the peripheral blood of CLL patients [5, 10]. MRFCs show diminished pokeweed mitogen responses and secrete autoantibodies of the IgM type characteristic of autoimmune disorders [8, 18, 29]. This explains similarities between MRFCs and the so-called "autoregulatory" B lymphocytes described by some authors [18, 32].

Leu 1 (CD 5) monoclonal antibody detects a lymphocyte membrane glycoprotein originally thought to be specific to mature T lymphocytes. Leu 1 (CD 5) can also be expressed by foetal spleen B lymphocytes and some B-cell lymphomas,

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especially by CLL B lymphocytes [1, 2, 16]. Leu 1 (CD 5) positivity indicates either immaturity of the B lymphocytes or the presence of a distinct subpopulation equivalent to "autoregulatory" B lymphocytes [6, 19].

5'-nucleotidase (5'-NU-ase) and *adenosine triphosphatase* (ATP-ase) are lymphocyte surface enzymes (ectoenzymes) of purine nucleotide metabolism [7, 9]. 5'-NU-ase activity is lower and ATP-ase activity is higher in CLL patients than in controls [11, 17]. The expression of 5'-NU-ase shows some correlation with cell maturation, i.e. in mitogen stimulated lymphocytes and myeloma cells 5'-NU-ase activity is higher than in immunodeficiencies and CLL [20, 23, 25, 28].

Pokeweed mitogen stimulation (PWM) results in polyclonal activation of B lymphocytes dependent on T-cell regulatory effects [15, 30]. Decreased PWM responses can be detected in CLL and in isolated MRFCs [18, 26]. Results of PWM responses in SLE are contradictory as both hyporeactivity and hyperreactivity of B lymphocytes have been reported [8, 30]. Above markers are widely studied in haematological disorders and in autoimmune diseases, especially in CLL and SLE.

B-cell chronic lymphocytic leukaemia (CLL) as a clonal proliferation of B lymphocytes is known to be a relatively slowly progressing lymphoproliferative disorder. The immature CLL B lymphocytes fail to develop into mature immunoglobulin secreting plasma cells. The exact mechanism of this maturation defect, the role of B-T-cell interactions, the immunoregulatory role of T lymphocytes in this condition has been widely discussed by several authors [4, 6, 14, 21, 22]. Primary immunoregulatory anomalies of B lymphocytes independent of T-cell regulatory effects have also been suggested. B lymphocytes in CLL expressed Leu 1 (CD 5) and M-rosette positivity [4, 9, 11, 26]. 5'-NU-ase and PWM responses are diminished in CLL [11, 17, 26].

Systemic lupus erythematosus (SLE) is a polysystemic autoimmune disease associated with autoantibodies secreted by polyclonally activated B lymphocytes [27, 30]. The role of regulatory T lymphocyte subsets in SLE is well established (decreased level of suppressor T cells, T helper cell hyperreactivity) [12, 13, 24]. It is known that in SLE B lymphocytes may be expressed by Leu 1 (CD 5), M-rosette positivity [8, 19] and anomalous functions [32] characteristic of "autoregulatory" B cells.

In the present study we compared some markers of peripheral blood lymphocytes in 10 patients with CLL, in 8 with SLE and in 10 controls. Correlation between the markers, co-expression and disease specificity are discussed.

Materials and Methods

Patients: The diagnosis of CLL in 8 males and 2 females was based on lymph node biopsies, bone marrow and blood studies in 9 cases (7 pseudofollicular, 2 of diffuse type) and in one case on bone marrow and blood tests. The white blood cell count (WBC) was 11.0–45.0 G/l, the ages of the patients ranged

between 43 and 81 years (median age 66.8). At the time of the study 3 of the patients were in stage Rai I, and 7 in Rai II. None of them had received treatment during the last six months. The diagnosis of SLE in 2 males and 6 females was based on the ARA (American Rheumatic Association) criteria [29]. Four patients had been treated with 10–15 mg Prednisolone daily, but not in the last six months. Their ages varied between 35 and 65 years (median age 39.5). The WBC was 3.0–7.0 G/l. The ages of the 10 controls varied between 26 and 40 years (median age 31.5). Both sexes were represented in controls (6 males, 4 females). These patient groups were not age- and sex matched because of the clinical nature of these diseases.

Isolation of the cells: Lymphocytes were isolated from heparinized venous blood of CLL, SLE patients and controls by Ficoll–Paque[R] (Pharmacia) gradient centrifugation. After removing monocytes by their plastic adherence, T and non--T lymphocytes were separated from each other on a Ficoll gradient based on their rosette forming capacity with 2-aminoethyl-isothiuroniumbromide (AET) pretreated sheep erythrocytes (E-rosette). After removing attached sheep erythrocytes by 0.83% ammonium-chloride lysis, the E-rosette positive cells ("T lymphocytes") were further separated into T_G-positive and T_G-negative cells ("T suppressor" and "T helper lymphocytes") by the anti-ox-IgG antibody--rosette (T_G-rosette) method [22]. The E-rosette negative cells (" B lymphocytes") were further separated into M-rosette positive and M-rosette negative subsets using CBA mouse erythrocytes [18].

Cell cultures: Isolated subsets (E^+, E^-, ${T_G}^+$, ${T_G}^-$, MRFCs and M-rosette negative cells) were cultured (2×10^6 cell/ml in RPMI 1640, Wellcome) with 5 μl pokeweed mitogen (Gibco Lab.) in 100 μl microtitre plates (Nuch) in a CO_2--thermostat for 120 hours [14, 22]. Co-cultures were made by mixing 50–50 μl of cells from the isolated subpopulations stimulated with PWM [18]. Cell proliferation was determined by Romanowsky Giemsa staining and in the co--cultures by immunofluorescence analysis of intracytoplasmic immunoglobulins of the B cells [30].

Immunocytochemical reactions: The binding of monoclonal antibodies (MoAbs) Leu 1 (CD 5, Becton–Dickinson), OKT_3 (CD_3, Ortho) and B_1 (CD 20, Coulter) were studied on cytospin preparations (Shandon) by the indirect immunoperoxidase method [21]. As a second antibody, peroxidase-conjugated rabbit immunoglobulin to mouse immunoglobulins (DAKO) and 3-amino-9-ethylcarbazole (Ortho, kit chromogen) were used. Each incubation was carried out at room temperature for 60 minutes, and the cytospin preparations were counterstained with Mayer's haematoxylin. The enzyme reactions (5'-nucleotidase and adenosine triphosphatase) used in cryostat sections [31] were adapted to cytospin preparations. Adenosine-5'-monophosphoric acid disodium salt (AMP) or adenosine-5'-triphosphoric acid disodium salt (ATP) (Reanal) was used in Tris-maleat buffer (pH 7.2) supplemented with magnesium sulphate and lead nitrate.

Results

Figure 1 shows the labelling of lymphocytes of 10 patients with CLL, 8 with SLE and 10 controls. The WBC of CLL patients were significantly higher than those of SLE patients and controls (see Materials and Methods). The percentage of T cells determined by OKT_3 (CD 3) monoclonal antibody was lower in CLL (18–32%) than in SLE patients and controls (52–78%) (Fig. 1a, "panB+panT"). The percentage of Leu 1 (CD 5) positive lymphocytes in controls and T cells determined by E-rosettes or OKT_3/CD 3 were equal. In CLL and SLE the percentage of Leu 1 (CD 5) positive cells was similar, but it was higher than the percentage of E-rosette positive or OKT_3 (CD 3) positive T cells ($p < 0.001$–-0.05). This means that some of the Leu 1 (CD 5) positive cells were B cells in both CLL and SLE (Fig. 1a, "Leu 1"). The percentage of mouse rosette forming cells (Fig. 1a, "M-rosette") was high in CLL (42–57%) and very low in SLE patients and controls (4–9%). The percentage of M-rosettes and Leu 1 (CD 5) positivity among non-E-rosette forming cells (Fig. 1b, "isolated B") was significantly higher in CLL (average 73%) than in SLE (average 14%). Leu 1 (CD 5) positivity of B cells in SLE patients was higher than in controls; this latter difference was, however, not significant (Fig. 1b, "M-ros." and "Leu-1"). No correlation between the absolute lymphocyte counts and the markers of lymphocyte subpopulations in individual cases were found (data not shown in Figures).

Figure 2a presents a MRFC (M-rosette). Figure 2b shows MRFCs of the same patient, one of them (arrow) reacting with Leu 1 (CD 5), the other being negative (arrow head). Figure 2c demonstrates isolated MRFCs from CLL. Three of the cells reacted with Leu 1 (CD 5) (open arrows), the others were negative.

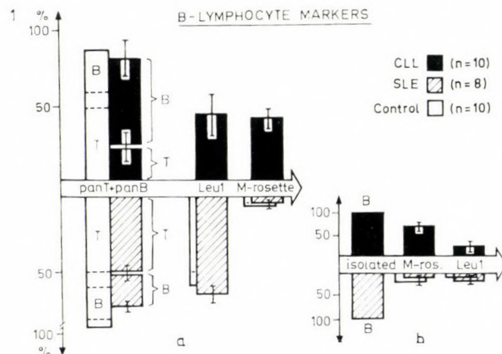

Fig. 1. B-lymphocyte markers in 10 cases of CLL, 8 cases of SLE and 10 controls. Distribution of T- and B-lymphocytes ("panT + panB"), Leu 1 and M-rosette positivity in peripheral blood lymphocytes (left side of diagram, 1a). M-rosette and Leu 1 positivity in isolated B-lymphocytes (insert, 1b). Standard deviations are shown at the top of the columns

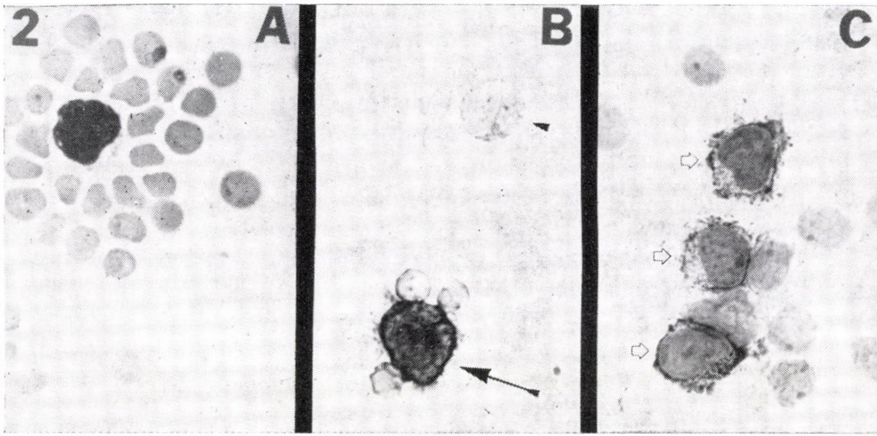

Fig. 2. A) M-rosette positive lymphocyte (cell with dark nucleus) forming rosette with CBA mouse erythrocytes (smaller gray anuclear cells attached). Romanowsky Giemsa staining. × 2000

B) M-rosette positive cells (two larger cells each with 3 attached erythrocytes), one of them showing Leu 1 positivity (cell at bottom with intense dark membrane positivity, arrow), the upper rosette forming cell is negative (gray, arrow head). Immunoperoxidase reaction. × 2000

C) Isolated M-rosette forming CLL B lymphocytes, three of them showing intense dark membrane positivity with Leu 1 monoclonal antibody (open arrows). The other cells (light gray) are negative lymphocytes. × 2000

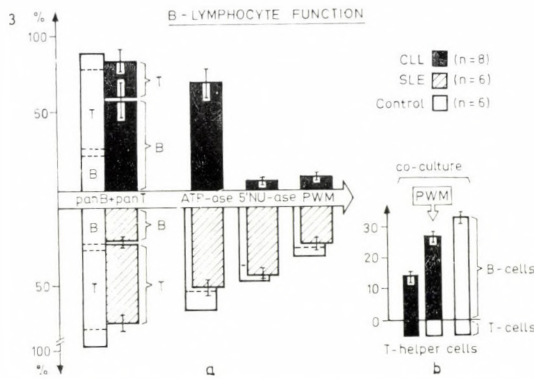

Fig. 3. B-lymphocyte function in 8 cases of CLL, 6 of SLE and 6 controls. Distribution of B- and T-lymphocytes ("panB + panT"), ATP-ase, 5'-NU-ase reaction and PWM stimulation in peripheral blood lymphocytes (left side of diagram, 3a). Co-cultures with B- and T-(helper)-cells in the course of PWM stimulation (insert, 3b). Standard deviation as in Fig. 1

In Fig. 3a, 8 patients with CLL, 6 with SLE and 6 controls are presented. The "panB + panT" values are similar to those shown in Fig. 1a. No significant differences were observed between the numbers of adenosine triphosphatase (ATP-ase) positive cells in CLL, SLE patients and controls (63–78 %). The 5'-NU--ase and pokeweed mitogen (PWM) responses were significantly decreased in patients with CLL ($p < 0.001$), compared to SLE and controls (Fig. 3a). Co--cultures (Fig. 3b) were grown with PWM stimulated "B-cells" (non-E-rosette forming cells) mixed with "T-helper cells" (IgG-F_c-receptor negative E-rosette forming cells). Normal T-helper lymphocytes (second column, Fig. 3b) had a greater stimulatory effect on CLL B lymphocytes than autologous T helper lymphocytes of CLL patients (first column, Fig. 3b) ($p < 0.01$). However, the values of normal B cells (third column, Fig. 3b) were not reached ($p < 0.02$).

Figure 4a shows PWM treated lymphocytes isolated from the peripheral blood of an SLE patient. The two larger cells (arrows) are blasts, the others are small lymphocytes. Figure 4b: In one of the PWM induced blasts from the same cell culture (open arrow) intense 5'-nucleotidase positivity is visible, while a small, non-stimulable lymphocyte is negative (black arrow). Figure 4c shows M-rosette positive B lymphocytes of a CLL patient. No blasts are visible after PWM stimu-

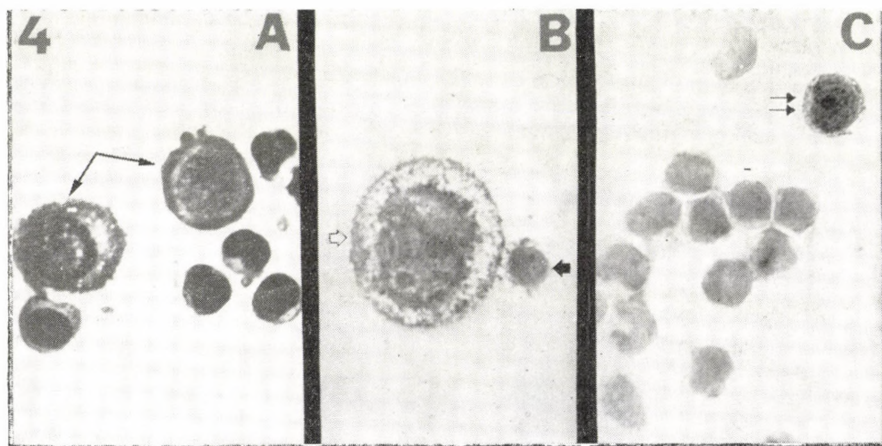

Fig. 4 A) Pokeweed mitogen (PWM) induced lymphocytes isolated from peripheral blood of SLE patient. Two larger cells show blastic transformation (arrow), the others are unresponsive small lymphocytes (smaller cells). Romanowsky Giemsa staining. × 2000

B) Pokeweed mitogen (PWM) induced lymphocytes from peripheral blood of SLE patient. Intense membrane enzyme positivity of a blast cell with 5'NU-ase (large cell with surface dark positivity, open arrow) and a negative, unresponsive small lymphocyte (black arrow) can be seen. Ecto-5'-nucleotidase reaction + pokeweed mitogen stimulation. × 2000

C) Isolated M-rosette forming CLL B lymphocytes. Unresponsiveness to PWM stimulation (no large blast cells, only small lymphocytes are present). Low 5'NU-ase reaction, (only one cell in top right corner shows granular dark membrane staining (double arrow). Ecto-5'-nucleotidase reaction + pokeweed mitogen stimulation. × 2000

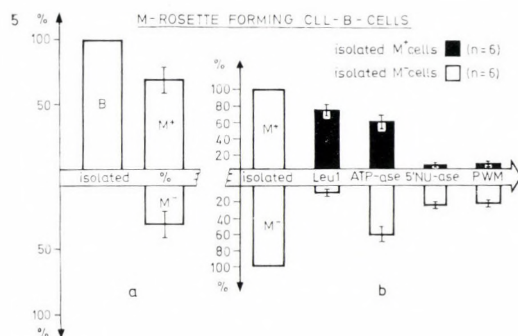

Fig. 5. M-rosette forming CLL B lymphocytes from 6 cases of B-CLL (isolated M^+ and M^- cells). Percentage of M^+ and M^- cells in isolated B-lymphocytes (left side of diagram, 5a). Leu 1, ATP-ase, 5'-NU-ase activity and PWM stimulation in isolated M^+ and M^- lymphocytes (right side of diagram, 5b). The deviation from standard values shown as in Fig. 1

lation, and except for one cell (double arrow) no 5'-nucleotidase positivity was observed.

In Fig. 5a isolated B lymphocytes (non-E-rosetting cells) from 6 patients with CLL were further isolated into M-rosette positive (52–76% of B cells) and negative cells. The Leu 1 (CD 5), ATP-ase, 5'-NU-ase positivity and PWM responses of isolated M-rosette forming and M-rosette negative cells are shown in Fig. 5b. The Leu 1 (CD 5) positivity is significantly higher ($p < 0.001$), while the 5'-NU-ase and PWM responses are diminished ($p < 0.01$) in M-rosette positive CLL B lymphocytes. Figure 5b shows no significant differences in ATP--ase activity.

Discussion

In the present study the expression of Leu 1 (CD 5), the number of MRFCs, purine-nucleotidase enzyme (5'-nucleotidase and adenosine triphosphatase) activities and pokeweed mitogen responses of lymphocytes from peripheral blood in CLL and SLE patients and in controls were measured. The reproducibility of these markers in the 10 cases of CLL was tested repeatedly in two-three months periods for two years (average positivity was 83–112%). The reported differences between T- and B lymphocyte markers and functions in older age groups [19, 25, 32] are not compatible with the significant changes we observed between CLL and SLE patient groups.

Leu 1 (CD 5) is a monoclonal antibody detecting a 67 kD membrane glycoprotein of peripheral T lymphocytes and a rather small number of B lymphocytes (autoregulatory B cells) [19]. Leu 1 (CD 5) positive B lymphocytes are present in the follicules of foetal lymph nodes and spleen and cord blood [1, 6, 16], in low grade malignant lymphomas (B-CLL and centrocytic ml) and in auto-

immune diseases [2, 6, 19]. Accordingly, our examination showed Leu 1 (CD 5) positivity of B lymphocytes in both CLL and SLE but not in controls (Fig. 1).

M-rosette (Fig. 2a) positivity, the spontaneous rosette formation of peripheral B lymphocytes with CBA mouse erythrocytes (mouse rosette forming cells, MRFCs) can be detected as a distinct minor subpopulation of normal peripheral B lymphocytes [18]. MRFCs are also present in cord blood and in peripheral blood after bone marrow transplantation [19]. High numbers of MRFCs can be observed in the peripheral blood of B-CLL patients [3, 5, 10]. Some authors suggest that autoregulatory B lymphocytes express Leu 1 (CD 5) and they are MRFCs [19, 32]. In this study SLE B lymphocytes expressed Leu 1 (CD 5) but did not form M-rosettes, while CLL B lymphocytes were positive for Leu 1 (CD 5) and formed M-rosettes (Fig. 1). Double labelling showed that not all MRFCs (Fig. 2b) were Leu 1 positive. However, isolated MRFCs in a CLL B lymphocyte population expressed significantly higher Leu 1 positivity (average 73%) (Fig. 2c) than did mouse rosette negative cells (average 9%) ($p < 0.001$).

Purine degradative enzymes, such as ecto-5'-nucleotidase (5'-NU-ase) and ecto-5'-adenosine-triphosphatase (ATP-ase) are present predominantly on normal B lymphocytes (but present at lower levels also on normal T lymphocytes), and can be observed in B cell malignancies [7, 23, 25]. The enzyme of 5'-NU-ase is deficient in B-CLL lymphocytes, and the activity of ATP-ase is higher [9, 11]. A reversal of this pattern (i.e. higher 5'-NU-ase and low ATP-ase activity) can be observed in malignant lymphoma cases showing plasmocytoid or plasmocytic differentiation [17, 23]. The lower 5'-NU-ase activity, as a differentiation marker might be a sign of immaturity in cases of infectious mononucleosis and in T suppressor lymphocytes of AIDS patients [25, 28]. Our results suggest that the significantly lower number of 5'-NU-ase positive B lymphocytes (Figs 3 and 5) are characteristic of CLL patients ($p < 0.001$). No alterations in 5'-NU-ase positive cells were seen in SLE patients compared to controls (41–53%). There were no significant alterations in ATP-ase activity of lymphocytes of either B-CLL or SLE patients compared to controls (63–73%).

Pokeweed mitogen response (PWM), a polyclonal activation of B lymphocytes dependent on T cell regulation, is characteristically diminished in B-CLL [14, 26] and in isolated MRFCs of controls [18]. The PWM response is in correlation with the functional immaturity of B cells in CLL [6, 15]. SLE B lymphocytes are polyclonally activated (the number of circulating immunoglobulin secreting cells is higher), while the responsiveness to PWM (and to T helper effects) may be defective [8, 30]. In the present study peripheral blood lymphocytes (Fig. 3) and isolated M-rosette positive CLL B lymphocytes (Fig. 5) had significantly decreased PWM responses ($p < 0.001$ and $p < 0.01$). SLE lymphocytes responded normally to PWM. In co-cultures (Fig. 3b) abnormalities of both T- and B-cell-responses were detected in the course of PWM stimulation in CLL patients. The blast cell response to PWM (Fig. 4a) and 5'-NU-ase positivity (Fig. 4b) showed parallelism, i.e. in SLE patients and controls both markers were normal, while CLL patients had decreased 5'-NU-ase activities and PWM responses (Fig. 3).

In isolated M-rosette positive CLL B lymphocytes (Fig. 4c) both 5'-NU-ase activity and PWM responses were diminished (Fig. 5).

Further studies of B lymphocyte markers are needed to understand the B cell differentiation and the pathogenesis of B cell lymphoproliferative and autoimmune disorders. Besides the general interest in using markers and functional studies of B lymphocytes in immuno-haemopoietic disorders, these observations also have a diagnostic significance.

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Does Human Bladder Carcinoma Cell Line 5637-Conditioned Medium Supplement the Growth of Megakaryocyte Colonies (CFU-Mk) in Cultures of Human Bone Marrow?

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Growth conditions of human megakaryocyte colony forming units (CFU-Mk) in vitro are still not clearly defined. Elaboration of reproducible culture conditions to promote growth of megakaryocyte colonies is important for the study of their development and differentiation in various diseases, and this must include a standard source of growth factors for CFU-Mk to replace plasma and medium conditioned by phytohaemagglutinin stimulated leukocytes (PHA-LCM) from donors.

The aim of this study was to compare the effect of human bladder carcinoma cell line 5637-conditioned medium (5637-CM) on megakaryocytopoiesis in healthy subjects with other stimulators such as: PHA-LCM, erythropoietin (EPO) and plasma from patients with aplastic anaemia (AA). The study was performed at the Dept. of Haematology University of Wales College of Medicine, Cardiff, U. K. Bone marrow samples were obtained with informed consent from haematologically healthy patients undergoing cardiac or orthopedic surgery. The CFU-Mk assay was as described by Messner et al. (1982) [1]. Plasma from healthy volunteers was added to PHA-LCM and EPO stimulated cultures. AA plasma was obtained from 11 patients and pooled. The effectiveness of conditioned media of CFU-Mk growth was assessed by the paired *t*-test. Table 1 shows a summary of actions of different stimulators on megakaryocyte colony formation in vitro. PHA-LCM promoted growth of CFU-Mk derived colonies to a greater extent after addition of EPO (1U/ml) ($p < 0.05$), but further increase of EPO to 2U/ml did not enhance this effect. 5637-CM alone stimulated CFU-Mk colony growth more effectively than PHA-LCM ($p < 0.005$). The CFU-Mk colony growth with 5637-CM was not statistically different from that in cultures

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Table 1

Effect of different stimulators on CFU-Mk derived colony formation in bone marrow from healthy subjects (n = 11)

Source of stimulators	CFU-Mk colonies per 2×10^5 cell culture		
	mean	SEM	range
Normal human plasma + PHA-LCM	16.1	6.9	9–35
Normal human plasma + PHA-LCM + EPO (1U/ml)	20.0*	11.0	11–48
Normal human plasma + PHA-LCM + EPO (2U/ml)	17.9	8.7	9–40
Normal human plasma + EPO (1U/ml)	19.3*	10.4	10–43
Normal human plasma + EPO (2U/ml)	14.6	8.9	7–36
5637-CM	22.9*	9.9	12–47
5637-CM + EPO (1U/ml)	21.9*	9.7	10–40
5637-CM + EPO (2U/ml)	18.2	7.3	10–37
AA plasma	26.6*	13.5	13–44

* $p < 0.05$ vs controls (Normal human plasma + PHA-LCM)

supplemented with AA plasma. The addition of EPO (1U/ml) to 5637-CM did not further stimulate CFU-Mk growth. A number of haemopoietic growth factors including megakaryocyte colony stimulating factor (Meg-CSF), recombinant (r) EPO and granulocyte/macrophage colony stimulating factor (GM-CSF) are capable of the stimulating cloning efficiency of human megakaryocyte progenitors [2]. Result of this preliminary study indicate that the bladder carcinoma cell line 5637-CM promotes the growth of CFU-Mk derived colonies to a similar extent as aplastic anaemia plasma. It was shown recently that 5637-CM contain factors which support the growth of erythroid, eoxinophil and granulocyte/macrophage progenitors, and also the growth of mixed pluripotent progenitors, all from normal human bone marrow [3]. Pluripotent CSF (pluripoietin alpha), one of two factors purified from human bladder carcinoma 5637 cell line-conditioned medium, might be responsible for this action [4]. Further study is required to elucidate the role of this factor in megakaryocytopoiesis and its interaction with other growth factors.

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Abstracts

Stimulation of human erythrocyte 2,3-bisphosphoglycerate phosphatase by vanadate. G. L. Mendz, S. J. Hyslop and P. W. Kuchel (Department of Biochemistry, The University of Sydney, New South Wales, Australia). *Arch. Biochem. Biophys. 276*, 160 (1990).

The rates of vanadate-stimulated hydrolysis of 2,3-bisphosphoglycerate in metabolically competent erythrocytes and in hemolysates were determined from data on time courses up to 35 min employing ^{31}P nuclear magnetic resonance spectroscopy. The enhanced rate of hydrolysis of the bisphosphate was attributed principally to the activation of the phosphatase activity of 2,3-bisphosphoglycerate synthetase both in cell suspensions and in hemolysates. Information on the concentrations of vanadate and vanadyl present in the preparations was obtained employing ^{51}V nuclear magnetic resonance spectroscopy and electron paramagnetic resonance spectroscopy. Redox reactions involving vanadium ions appeared to be important in establishing the final equilibrium concentrations of the oxy- and oxo-ions (vanadate and vanadyl, respectively), but the data suggested that the activation of the enzyme resulted from direct action of the vanadium ions on the enzyme and not as a consequence of the alteration in the equilibrium of intracellular oxidants and reductants.

Ágnes Enyedi

Fatty acid acylation of membrane skeletal proteins in human erythrocytes. D. Maretzki, M. Mariani and H. U. Lutz (Laboratory for Biochemistry, Swiss Federal Institute of Technology, ETH-Zentrum, Zurich, Switzerland). *FEBS Letters 259*, 305 (1990).

Fatty acid acylation of membrane proteins was studied on human erythrocytes by measuring incorporation of [^{3}H]palmitate at different specific radioactivities. A 55 kDa polypeptide within the band 4.5 region was the main acceptor protein for acylation by fatty acids (palmitate, stearate, oleate), while other polypeptides (80, 65, 48, 30 kDa) incorporated [^{3}H]palmitate slowly, in substoichiometric amounts. Integral membrane proteins were preferentially fatty acid acylated. Skeletal membrane proteins were, however, poorly labeled. Neither purified ankyrin nor band 4.1 protein were fatty acid acylated in human erythrocytes. On the other hand, label associated with high molecular weight skeletal proteins resisted low and high ionic strength extractions, and was extracted selectively by urea along with a small subpopulation of spectrin which was also tightly associated with the membrane.

Ágnes Enyedi

Age-related changes of erythrocyte membrane in the senescence-accelerated mouse. H. Abe and M. Orita (Research Institute of Oriental Medicine, Kinki University, Ohnohigashi, Osakasayamashi, Osaka, Japan). *Mechanisms of Ageing and Development 51*, 215 (1990).

Age-related changes in erythrocytes in senescence-accelerated mice (SAM-P) and control mice with normal aging characteristics (SAM-R) were examined. A significant decrease in the number of erythrocytes and significant increases in MCV and ATP levels were observed with aging in SAM-P, while no significant changes were seen in SAM-R. Erythrocytes in aged SAM-P were less fragile than those in aged SAM-R. The contents of cholesterol and phospholipids in erythrocyte membranes increased significantly in aged SAM-P, but the molar ratio of cholesterol/phospholipid decreased. The plasma cholesterol level of SAM-P decreased with aging. Changes such as those observed in SAM-P were not seen in SAM-R during the period of observation.

Ágnes Enyedi

Carriers of ataxia-telangiectasia gene display additional protein fraction and changes in the environment of SH groups in erythrocyte membrane. M. Rybczynska, A. L. Pawlak, S. K. Hoffmann and R. Ignatowicz (Department of Biochemistry, Medical Academy, Poznan, Poland). *Biochim. Biophys. Acta 1022*, 260 (1990).

Additional protein fraction migrating slower than spectrin has been detected in erythrocyte membranes from an ataxia-telangiectasia (A-T) patient and from his mother (A-T heterozygote). In erythrocyte membranes labelled with maleimide spin label changes in signal of the weakly immobilized spin label as related to that of strongly immobilized one (w/s) were noted. In comparison to age-matched control groups the values of w/s were lower in A-T heterozygotes (ten persons) and higher in A-T homozygotes (four persons). In control persons the values of w/s increased with age, whereas in families with A-T no significant differences in this parameters were noted between children and parents. The presence of additional protein fraction in erythrocytes membranes of A-T patient and A-T heterozygote indicates that these phenotypes can be differentiated from the healthy control persons for the first time on the basis of changes detected in the erythrocytes. This change in erythrocyte membrane may explain the decrease in the w/s parameter of electron spin resonance in A-T heterozygotes. On the other hand increased values of w/s in A-T patients may be caused by disease process.

G. Gárdos

Reduced intracellular pH in lymphocytes from the spontaneously hypertensive rat. D. C. Battle, A. Saleh and G. Rombola (Northwestern University Medical School and the Lakeside Veterans Administration Medical Center, Chicago, Illinois, USA). *Hypertension 15*, 97 (1990).

This study was designed to determine the cytoplasmic pH (pH_i) profile of lymphocytes from a rat model of genetic hypertension that is well suited for study before and after the development of spontaneous hypertension. For this purpose, pH_i was measured in thymic lymphocytes obtained from spontaneously hypertensive rats (SHR) and from age-matched Wistar-Kyoto (WKY) control rats using 2',7'-bis carboxyethyl-5,6-carboxyfluorescein (BCECF), a pH-sensitive fluorescence probe. At the age of 16–20 weeks, pH_i of lymphocytes suspended in a HCO_3-free HEPES-buffered solution, was markedly lower in the SHR than in the WKY rats (7.07 ± 0.02, $n = 16$ and 7.22 ± 0.01, $n = 15$, respectively, $p < 0.001$), whereas systolic blood pressure was higher in SHR than in WKY rats (175 ± 5.0 and 105 ± 3.0 mm Hg, respectively, $p < 0.001$). In rats less than 5 weeks of age, pH_i was also lower in SHR than in WKY rat lymphocytes (7.12 ± 0.04, $n = 11$ and 7.23 ± 0.04, $n = 11$, respectively, $p < 0.05$), although at this age systolic blood pressure was not different

between the two groups (87 ± 4.0 and 85 ± 3.0 mmHg, respectively). In lymphocytes suspended in a more physiological HCO_3/CO_2-buffered solution, pH_i was again lower in the adult SHR than in the WKY rat (7.18 ± 0.02, $n = 16$ and 7.31 ± 0.02, $n = 16$, respectively, $p < 0.001$). Under this condition, blockade of $Na^+ - H^+$ exchange by a specific inhibitor, ethyl-isopropyl-amiloride (EIPA), or by exposure to Na^+-free media resulted in intracellular acidification in both SHR and WKY rat lymphocytes. The difference in pH_i between SHR and WKY rat lymphocytes persisted after blockade of the $Na^+ - H^+$ exchanger by either EIPA (7.09 ± 0.04 vs. 7.20 ± 0.03, $p < 0.05$) or by removal of external Na^+ (7.04 ± 0.05 vs. 7.19 ± 0.02, $p < 0.005$). These data show that reduced pH_i is a feature of lymphocytes from the SHR that is apparent before and after the development of hypertension. Persistence of reduced pH_i in SHR cells, as compared with WKY rat cells, after $Na^+ - H^+$ blockade suggests that a primary abnormality in the activity of the $Na^+ - H^+$ exchanger is unlikely to account for the observed reduction in steady-state pH_i of SHR cells. This alteration in pH_i regulation persisted after removal of external Na^+ and was seen in the presence and in the absence of external HCO_3, indicating that a sodium-independent, bicarbonate-independent mechanism of cytoplasmic acid accumulation accounts for the observed difference in pH_i.

B. Sarkadi

Antibodies against membrane interleukin 1α activate accessory cells to stimulate proliferation of T lymphocytes. E. M. Eugui and S. J. Almquist (Department of Immunology, Syntex Research, Palo Alto, CA., USA). *Proc. Nat. Acad. Sci. USA. 87*, 1305 (1990).

Some monoclonal antibodies (mAbs) against interleukin (IL) 1α have been found to activate antigen-presenting cells (APC, human peripheral blood monocytes and B lymphocytes), so that unstimulated T lymphocytes cultured with them are induced to proliferate and secrete IL-2. Control mAbs of the same isotypes and mAbs against IL-1β do not activate APC. In the absence of APC, mAbs against IL-1α do not induce proliferation of T lymphocytes. Mitomycin C-treated activated APC still induce T-cell proliferation. Proliferation of T lymphocytes cannot be induced by culture supernatants and requires contact with APC activated by mAbs against IL-1α. The observations imply that surface membrane IL-1α can function as a triggering molecule on APC, which could play an important role in the initiation of immune responses by T lymphocytes.

B. Sarkadi

Patch-clamp profile of ion channels in resting murine B lymphocytes. F. V. McCann, D. C. McCarthy and R. J. Noelle (Department of Physiology and Microbiology, Dartmouth Medical School, Hanover, New Hampshire, USA). *J. Memb. Biol. 114*, 175 (1990).

Patch-clamp studies of single ion channel currents in freshly isolated murine B lymphocytes are characterized here according to their respective unitary conductances, ion selectivities, regulatory factors, distributions and kinetic behavior. The most prevalent ion channel in murine B lymphocytes is a large conductance (348 pS) nonselective anion channel. This report characterizes additional conductances including: two chloride channels (40 and 128 pS), a calcium-activated potassium channel (93 pS), and an outwardly rectifying potassium channel which displays two distinct conductances (18 and 30 pS). Like the anion channel, both chloride channels exhibit little activity in the cell-attached patch configuration. The kinetic behavior of all of these channels is complex, with variable periods of bursting and flickering activity interspersed between prolonged closed/open intervals (dwell times). It is likely that some of these channels play an important role in the signal transduction of B cell activation.

B. Sarkadi

ADP evokes biphasic Ca^{2+} influx in fura-2-loaded human platelets. Evidence for Ca^{2+} entry regulated by the intracellular Ca^{2+} store. S. O. Sage, R. Reast and T. J. Rink (The Physiological Laboratory, University of Cambridge, Cambridge, UK). *Biochem. J. 265,* 675 (1990).

Stopped-flow fluorimetric studies at 37 °C have shown that ADP, at optimal concentrations, can evoke Ca^{2+} or Mn^{2+} influx in fura-2-loaded human platelets without measurable delay. In contrast, the release of Ca^{2+} from intracellular stores is delayed in onset by about 200 ms. By working at a lower temperature, 17 °C, the rise in cytosolic calcium concentraton ($[Ca^{2+}]_i$) evoked by ADP in the presence of external Ca^{2+} is biphasic. The use of Mn^{2+} as a tracer for bivalent-cation entry indicates that both phases of the ADP-evoked response are associated with influx. The fast phase of the ADP-evoked rise in $[Ca^{2+}]_i$, which occurs without measurable delay at both 17 °C and 37 °C, is consistent with Ca^{2+} entry mediated by receptor-operated channels in the plasma membrane. The delayed phase, indicated by Mn^{2+} quench, is coincident with the discharge of the intracellular Ca^{2+} stores. Forskolin did not inhibit the fast phases of ADP-evoked rise in $[Ca^{2+}]_i$ or Mn^{2+} quench, but completely abolished ADP-evoked discharge of the intracellular stores, the delayed phase of the rise in $[Ca^{2+}]_i$ observed in the presence of external Ca^{2+} and the second phase of Mn^{2+} quench. The timing of the delayed event appears to be modulated by $[Ca^{2+}]_i$: the delayed phase of Mn^{2+} quench coincides with discharge of the intracellular stores in the absence of added Ca^{2+}, but with the second phase of the ADP-evoked rise in $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}. Similarly, blockade of the early phase of Ca^{2+} entry by SK&F 96365 further delays the second phase. It is suggested that a pathway for Ca^{2+} entry which is regulated by the intracellular Ca^{2+} store exists in platelets. This pathway operates alongside, and appears to be modulated by the activity of, other routes for Ca^{2+} entry into the cytosol.

G. Gárdos

How does fluoroaluminate activate human platelets? F. Rendu, M. Lebret, D. Tenza and S. Levy-Toledano (U 150 Inserm, URA 334 CNRS, Hôpital Lariboisière, 75010 Paris, France). *Biochem. J. 265,* 343 (1990).

Platelet activation induced by NaF or fluoroaluminate (AlF_4^-) was studied. The latter has been described to substitute for the γ-phosphate group of the GTP molecule. With 10 mM-NaF, a concentration unable to induce any measurable Ca^{2+} mobilization (as measured with Indo 1), addition of $AlCl_3$ potentiated platelet aggregation, thromboxane synthesis, diacylglycerol formation and p43 phosphorylation, without any increase in intracellular Ca^{2+}. Neither phosphoinositide hydrolysis nor phosphatidic acid formation could be detected. AlF_4^- induced the release through a granule centralization within a microtubule bundle, although no myosin light-chain phosphorylation could be detected. Addition of flurbiprofen (10 μM) resulted in only partial inhibition of diacylglycerol formation, with no effect on the release reaction or on p43 phosphorylation. The present results suggest that AlF_4^- does not stimulate a G-protein governing the phosphoinositide-specific phospholipase C. The AlF_4^--induced diacylglycerol formation is discussed. Moreover, these results bring evidence that there is no correlation between granule centralization and myosin light-chain phosphorylation.

G. Gárdos

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How T Cells Talk to Target Cells*

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T cell defects are very often involved in haematological diseases and occur even more frequently as a consequence of our therapeutic interventions. T cells play a central role in the immune defence of the organism. To perform this function, they move around the body to achieve a particular stage of development and in search for antigenic insult. In the course of this, they have to communicate and cooperate with various cells. T cells carry a high number of structurally and functionally defined and even more as yet undefined surface structures. The variability and functional pleitropism of these molecules and as a consequence that of their interactions with surface structures of other cells is infinite. This paper will be limited to discuss briefly the following subjects:

- the presentation of antigens to T cells
- structure and function of the T cell receptor (TCR) and its interactions with the antigen presenting major histocompatibility complex (MHC) molecule
- accessory molecules and other modulating surface structures
- the role of carbohydrates
- pitfalls of inferences on interacting cell surface structures

Keywords: T cells, target cells, major histocompatibility complex, carbohydrates, T cell receptor, antigens

The Presentation of Antigens to T Cells

Whereas immunoglobulins (Ig) have both soluble and membrane forms that can bind to antigen alone, antigens have to be presented in an attractive form to T cells (Fig. 1). The specific antigen receptor of T cells exists only on cell surfaces and recognizes only processed antigen fragments embedded in the MHC class I or class II antigens. The antigen presenting cell (APC) for regulatory cells may be a MHC class II positive interdigitating cell, a B cell or a professional macrophage. Any MHC class I positive cell can function as an APC for cytolytic T cells (CTL). The presented antigenic fragments are short peptides. The fact that amino acid substitutions far from the antigenic determinant can modulate T cell recognition, points to the conformational recognition of a large peptide.

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VSP, Utrecht, Tokyo
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The antigen has to be presented to T cells.

Fig. 1. Antigens have to be presented in an attractive manner

The complete steric structure of the MHC class I HLA-A2 molecule had been disclosed by the X-ray crystallographic studies of Björkman et al. (1987). The HLA-A2 molecule consists of two pairs of structurally similar domains:

1) the membrane-distal α-1 and α-2 domains and

2) the membrane-distal α-3 and β_2 microglobulin and α-3 domains.

The α-1 and α-2 polypeptide chains form a deep groove on the top of the HLA-molecule (Fig. 2), the bottom of which is lined by anti-parallel β-pleated sheets spanned by the two long α-helices (Fig. 3). This is in fact the specific antigen binding site of the MHC molecule. Most of the non-conserved residues are located in this site, selected to generate an ability to present many different peptides. Conserved residues pointing into the recognition site serve probably as binding sites for TCR and other T cell epitopes. Björkman et al. found processed antigenic peptides bound to the antigen recognition site. The fact that these remained bound to HLA during the entire purification and crystallization procedure

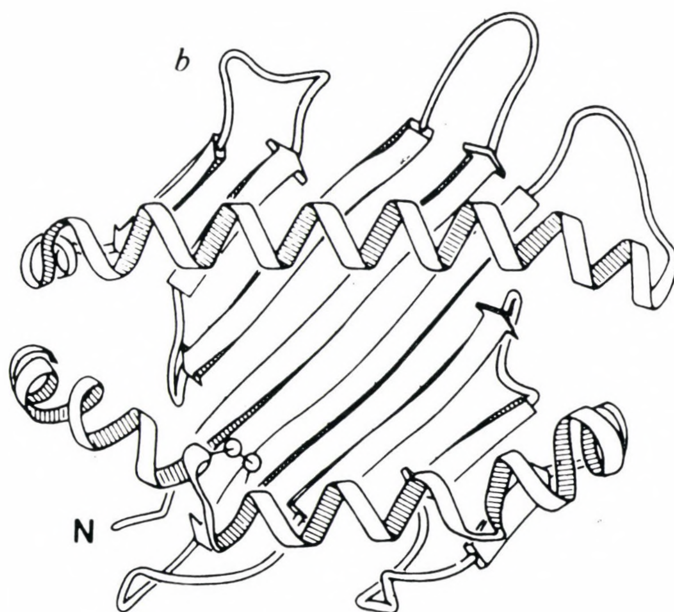

Fig. 2. The two pairs of structurally similar domains of the HLA-A2 molecule
(P. J. Björkman et al.: Structure of the human class I histocompatibility antigen. HLA—A2. Reprinted by permission from NATURE Vol. 329, p. 508, Copyright © 1987 Macmillan Magazines Ltd.)

argues for a very slow kinetic rate off for peptides bound to HLA molecules. It is surmized that in the absence of foreign antigens, processed peptides of self antigens occupy the binding site.

Structure and Function of the TCR and Its Interactions with the Antigen Presenting MHC Molecule

In contrast to the precise steric structure of the MHC-Ag complex, very little is known at the molecular level on how the TCR recognizes this complex.

The TCR-s perform the complex function of 1) recognition of specific antigens and 2) the translation of antigen binding into biochemical signals that activate the immune response of T cells. This multi-unit receptor is present on all mature and many developing T cells. The TCR is comprised of the antigen recognition part Ti, which is stoichiometrically but non-covalently associated with the invariant proteins of the CD3 complex (Fig. 4). The Ti is built up by polymorhic α/β or γ/δ chains, the α/β heterodimer being far more common. In normal individuals TCR γ/δ is found on 1 to 10% of mature CD3 pos. peripheral blood T cells, on the majority of dendritic T cells of the skin and on 0.1 to 1.0%

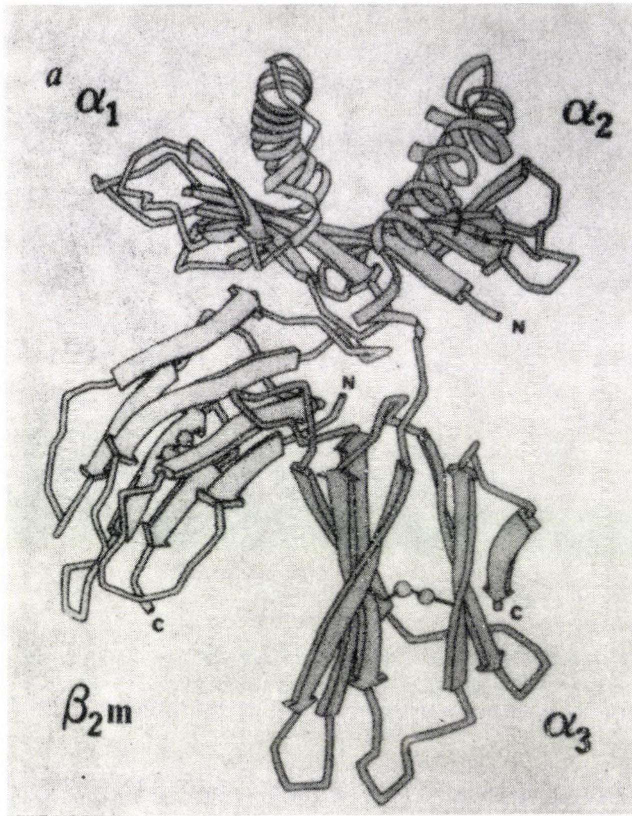

Fig. 3. The antigen binding site of the MHC molecule
(P. J. Björkman et al.: Structure of the human class I histocompatibility antigen. HLA—A2. Reprinted by permission from NATURE Vol. 329, p. 509, Copyright © 1987 Macmillan Magazines Ltd.)

of the most immature CD3 pos. thymocytes. The human CD3 is comprised of γ, δ, ε and disulfide-linked ξ-chains. The large intracellular portion of CD3 chains as compared to Ti chains (which have large extracellular domains and only 5 to 12 intracellular amino acids) points to the transmembrane signalling function of the CD3 complex. This is especially true for the ξ-chain which has an extremely small extracellular and a very large intracellular domain with 6 potential sites for tyrosine phosphorylation. The tyrosine-phosphorylated ξ-chain is the previously described p21, the protein known to become tyrosine-phosphorylated in response to Ag binding. The recently described η-chains, are linked to approximately 10% of the ξ-chains. The lack of η-chains has been described to result in inefficient coupling of receptor occupancy to phosphoinositide (PI) hydrolysis (Marcep et al. 1988).

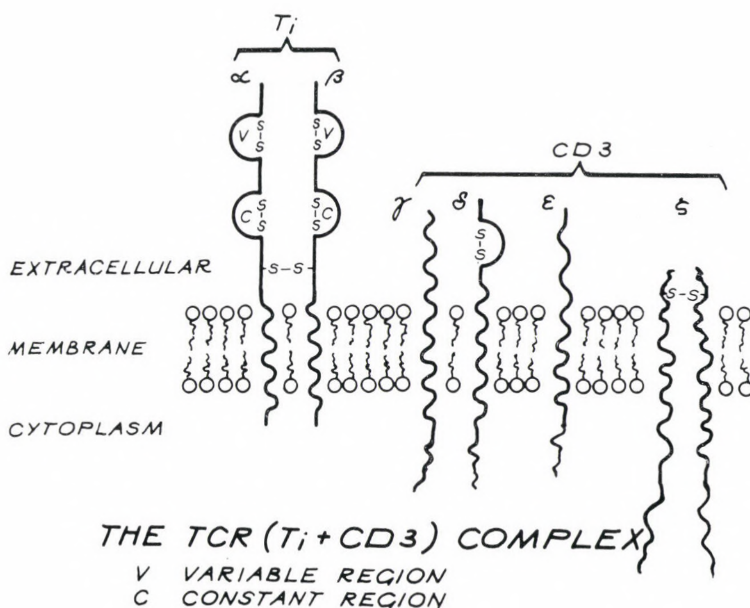

Fig. 4. The TCR (Ti + CD3) complex

The transmembrane region of all CD3 components have a charged residue in their completely hydrophobic transmembrane sequence: γ-, δ- and ε-chains have a positively charged, while the ξ-chain a negatively charged residue. This very exceptional feature — to have non-covalent interactions within the transmembrane domain of each CD3 subunit — may enable the complex CD3 structure to react on binding of even small doses of antigen to Ti with an allosteric shift, that is magnified through the complex, ending up in a second stable conformation in which multiple contacts with the second messenger system are activated.

To get a better insight into details of TCR/Ag/MHC interactions, Davis and Björkman (1988) summarized current structural information on TCR genes and their products and offered a hypothetical structural model, while Claverie et al. (1989) constructed a complete steric model of the TCR molecule and investigated the putative organization of the TCR/peptide/HLA-A2 complex. Both models are based on the significant homology between the antigen-contacting variable domains of the Ig heavy and light chains (VH and VL) and the variable domains of the TCR (V_α and V_β) with an average of 25 – 40 % amino acid identity. TCR-s are thus suggested to have similar spatial organization to the Ig Fab moiety. The TCR V_α and V_β regions have just like V_H and V_L, three variable loops designed to make contact with the antigen and are thus referred to as complementarity-determining regions, CDR1, CDR2 and CDR3 in both mole-

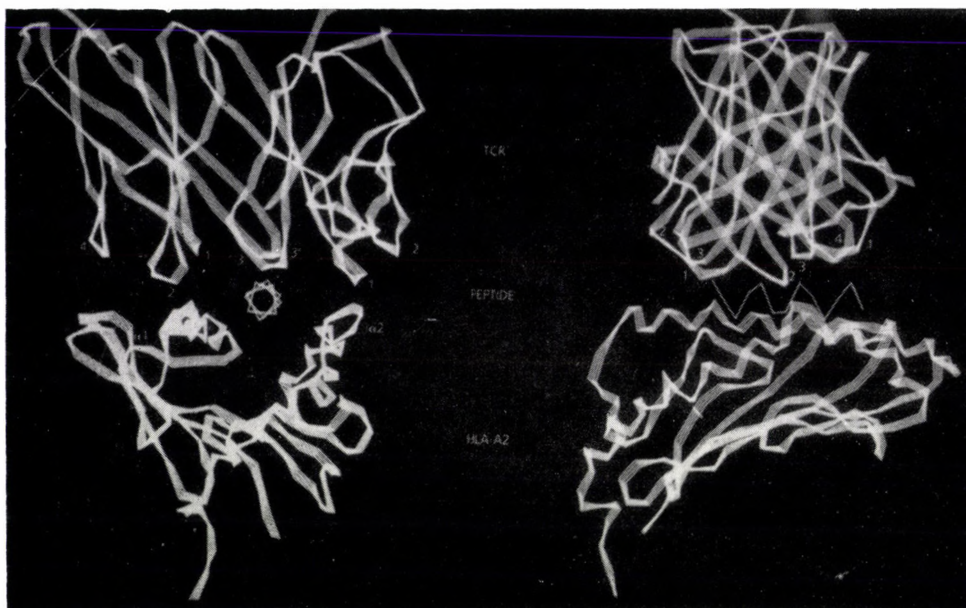

Fig. 5. A speculative model of the TCR/peptide/HLA-A2 interaction
(J.-M. Claverie et al.: Implications of a Fab-like structure for the T-cell receptor. Reprinted by permission from IMMUNOLOGY TODAY Vol. 10, p. 12. Copyright © 1989 Elsevier Trends Journals)

cules. The respective geometrics of a Fab-like TCR structure and of the HLA-A2 binding site suggest a model where both variable CD3 chains of TCR interact with the peptide antigen presented at the antigen binding site of the MHC molecule. The first and the second less variable regions (CDR1 and CDR2) of the TCR molecule are in a position for making contact with both polymorphic and conserved residues pointing up from the α-1 and α-2 helical regions of the antigen presenting HLA molecule (Fig. 5). If the peptide can fit deep inside the groove, the interactions between the TCR CDR1/CDR2 and the HLA-A2 can be realized. If the peptide protrudes into the interface of the contacting molecules, steric constraints could preclude the interactions between the MHC and TCR molecules. This is the molecular basis of the MHC restriction that cannot be easily reconciled with totally antigen-independent MHC-restriction schemes as that i.e. of the self-"learning" hypothesis.

Accessory Molecules and Other Modulating Surface Structures

The presentation of peptides by MHC class I or class II molecules seems to correlate with the endogenous (live virus, self-proteins) versus exogenous (and thus first endocytosed) nature of the native antigen. There are no essential

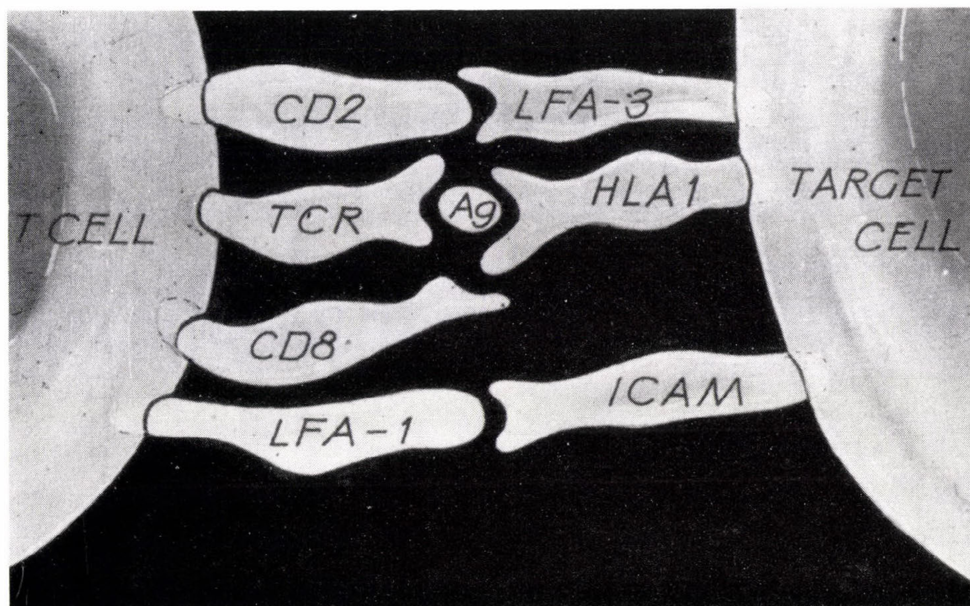

Fig. 6. Molecules crucial to the activation of T cells are all members of the immunoglobulin superfamily of adhesive proteins

features distinguishing these two pathways at the level of peptide binding or for the type of TCR involved in antigen recognition. The basic differences between helper T cells (mostly reacting with MHC class II presented antigen) and CTL-s (mostly reactive to MHC class I presented antigen) is thought to depend on the interaction with the surface molecules CD4 and CD8.

CD4 and CD8 molecules, just like all other surface molecules crucial to T lymphocyte activation (subunits of TCR, class I and class II MHC, CD2, leukocyte function associated molecules LFA-1 and LFA-III), are all members of the Ig superfamily. All these molecules share not only structural similarities between their disulphide bonded extracellular domains but have also a common adhesive function (Fig. 6). The regulatory interactions between cell adhesion molecules concentrate actions to restricted areas of the lymphocyte membrane amplifying in this manner weak reactions. T cells reorient their microtubule-organizing centres and Golgi apparatus towards the site of interaction with APC-s (Poo et al. 1988). T cells and APC-s can thus form transient mobile synapses for the bidirectional exchange of critical information. Lymphokines and other cytokines are secreted into these synaptic spaces via directional exocytosis.

Both the induction of T helper cells by APC-s and the delivery of help to B cells need direct contact (Fig. 7). Similarly, the cytotoxic activity of T cells is dependent on an appropriate contact between the CTL and its target cell. The initial adhesions are mediated via LFA-1 and CD2 molecules (Fig. 8). These

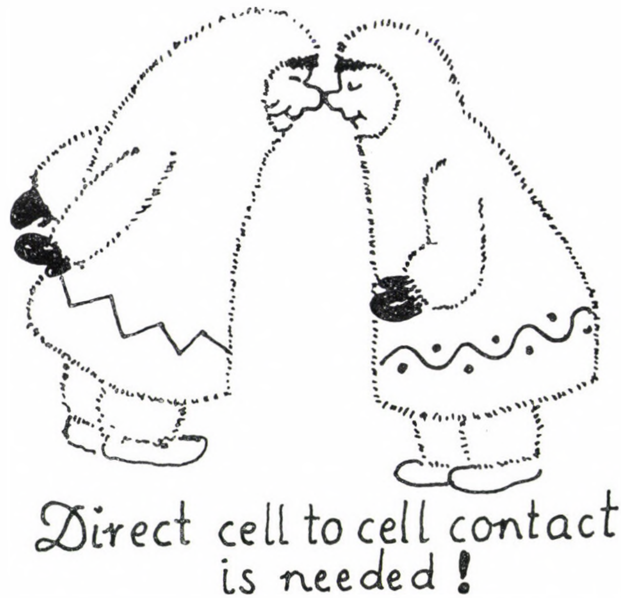

Fig. 7. Direct cell to cell contact is needed for interactions of T cells with antigen presenting cells

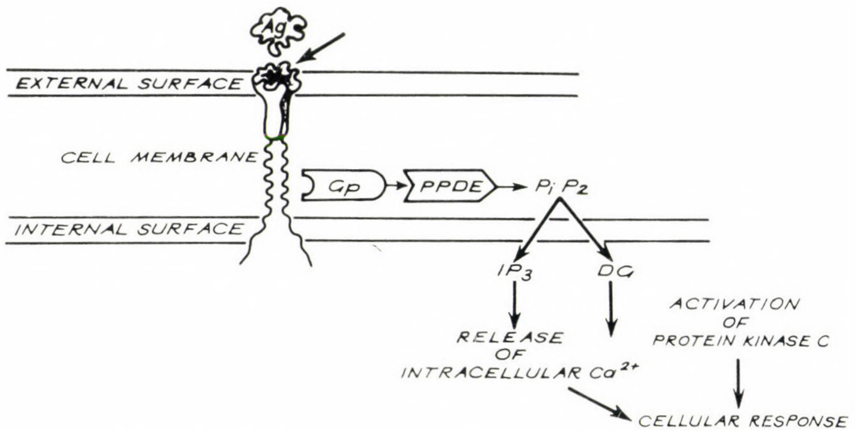

Fig. 8. Antigen-dependent and antigen-independent pathways of T cell activation

antigen non-specific interactions and the resulting antigen-independent activation of the T cells were shown to precede the recognition of Ag by TCR. The strong association between CD2 and LFA-3 (binding constant 1.7×10^{-7} M) may be necessary to stabilize adhesions between motile cells. Similarly LFA-1 can interact (among other ligands) with the intercellular adhesion molecule (ICAM-1) which like LFA-3 has a broad tissue distribution. CD2 – LFA-3 and LFA-1 – ICAM-1 binding provide the T cell with two different adhesion pathways which differ in their temperature dependence and Mg^{2+} requirements (de Vries et al. 1989). The use of a certain adhesion pathway may result in different physiological responses of T cells. Apart of CD2 and LFA-1 some other surface structures of human T cells (CD28 and Tp 103) are claimed to have the capacity to activate in a non-specific way. In mature T cells the antigen-dependent and independent pathways are able to interact, which may reflect a system of checks and balances necessary to maintain homeostasis in the immune system.

CD4 and CD8 are also involved in adhesions between T cells and APC-s and in adhesions between CTL-s and target cells, respectively. CD8 and CD4 are considered as minor adhesion molecules as compared to CD2 and LFA-1 (de Vries et al. 1989). CD4/CD8 molecules are needed to TCR antigen interactions in situations where the intrinsic affinity of the TCR for its antigen is low, or in cases where only low concentrations of antigen are accessible for the TCR. Biddison and Shaw (1989) point out that surface molecules situated in such a manner as to mediate adhesion are also ideally suited for transducing information. CD4 and CD8 molecules not only serve to enhance the avidity of T cells by binding to class II and class I MHC antigens, but are also involved in the post binding events of CTL activation. Recent studies suggest that CD4 and CD8 play a critical role in the intrathymic development of T cells as well. CD4 and CD8 are proposed to be bifunctional molecules capable of interacting with MHC class I and class II molecules on stimulator and target cells, and with the TCR-s on the same cell. Depending on the state of differentiation, one or the other binding function is more important (Eichmann et al. 1989).

Veilette et al. (1988) have shown that a src-related tyrosine-kinase, the p56 is associated with the cytoplasmic parts of CD4/CD8. Through activation of the Ti – CD3 complex, the adhesion of CD4/CD8 to TCR may bring $p56^{lck}$ to direct contact with the ξ-chain resulting in its tyrosine phosphorylation (Fig. 9). This is how the physical association of CD4/CD8 with an intracellular kinase is involved in transmembrane signalling of T lymphocytes. Mustelin and Altman (1989) suggested that CD4/CD8 may control the mobility and hence the accessibility of $p56^{lck}$ to the ξ-chain in the T cell membrane.

It is still a wide-spread dogma that CD4 expression is strictly correlated with helper function and that CD4 reacts restrictedly with MHC class II antigen, while CD8 positive cells are strictly suppressor or cytotoxic cells reacting selectively with MHC class I antigens. The initially oversimplified categorization of suppressor T cells became recently one of the most controversial topics in immunology. The inability to correctly identify suppressor cells as a distinct T cell

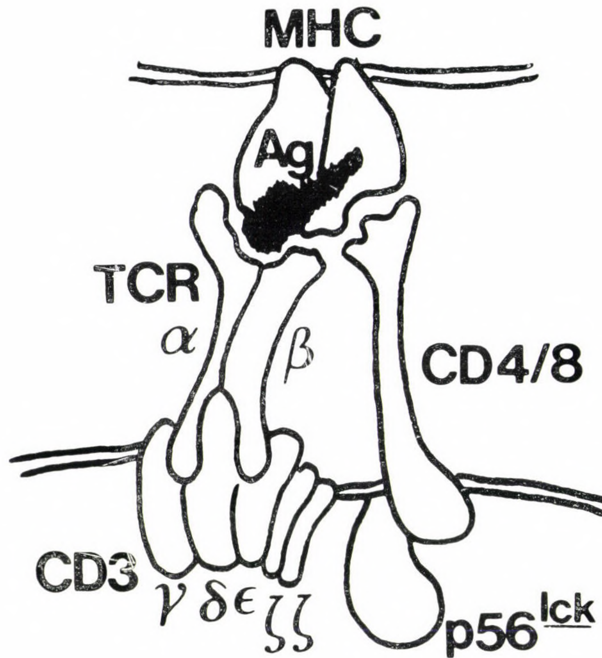

Fig. 9. Model of MHcl/Ag/TCR, CD4 or CD8 and p56^{lck} aggregate.
(T. Mustelin and A. Altman: Do CD4 and CD8 control T-cell activation via a specific tyrosine protein kinase? Reprinted by permission from IMMUNOLOGY TODAY Vol. 10, p. 190, Copyright © 1989 Elsevier Trends Journals)

subset has led to the conclusion that they simply may not exist (Möller 1988). It is now accepted that class II restricted T cells are heterogenous at the functional level (helper and cytotoxic) and they are also – albeit rarely – heterogenous at the phenotypic level (CD4 and CD8). The basis of this heterogeneity is as yet unresolved. There are different possibilities: 1) two separate MHC class II restricted T cell lineages may exist which may only differ in their requirements for lymphokines and/or accessory signals, 2) the intensity of the triggering stimulus may determine the effector function, or 3) the two functions may be performed by T cells at different stages of development. Lanzavecchia (1989) raises the hypothesis that the MHC-class II restricted CTL-s might be the endlessly elusive T suppressor cells. De Vries et al. (1989) have described that interleukin-4 (IL-4) can induce the expression of CD8+ protein on CD4+ cells, illustrating that the phenotype of mature functional CD4+ cells is not irreversibly fixed. IL-4 may also be associated with the induction of CD8 expression during the transition of CD4 CD8 to CD4+CD8+ thymocytes during thymic maturation.

Whereas migration of non-activated lymphocytes is widespread through the body, effector cells and memory cells exhibit remarkable selective migratory

behaviour, moving mainly to tissues originally involved in antigen exposure and cell activation. The extravasation of blood-borne lymphocytes is regulated by interactions of the address-dependent expression of homing receptors with the endothelium of specialized organ venules, the so-called high endothelial venules (HEV). At least three independent homing receptor systems are responsible for controlling traffic of the lymphocytes to different areas of the lymphoid system and to inflamed synovia. Homing receptors are also expressed by many human lymphoid neoplasms and appear to play a role in lymphoma metastasis.

A superfamily of endothelial surface molecules selectively expressed on mucosal organs are the ligands for lymphocyte homing receptors. These 'vascular addressins' appear to function as tissue specific address signals for recognition by lymphocytes in the circulating blood (Streeter et al. 1988). Rosen et al. (1985) suggested that sialic acids on endothelial cells may be organ specific recognition determinants for lymphocyte attachment. Lymphocytes bear also receptors which recognize fibronectin, collagen, laminin, fibrinogen and von Willebrand factor. The fibronectin receptors appear to be essential in the colonization of the thymus by T cell precursors. Adhesion capability of more differentiated T cells is diminished or absent. Fibronectin receptors are thought to anchor the developing cells until they are ready to emigrate. The difference between the expression of various adhesive protein receptors also reflects the different matrix adhesion requirements of mobile, stimulated cells versus quiescent cells (Hemler 1988).

Six molecules (CD2, LFA-3, LFA-1, UCHL1, Pgp-1, CD44 and CDw29) are all either newly expressed or increased in expression on memory T cells. Since Pgp-1 in man is the Hermes-homing receptor, homing receptors as well as ICAM-1 and VLA molecules are also increased on memory cells. The heightened level of many adhesion molecules on memory T cells suggest that facilitated cell interactions may account for at least some of the features of secondary immune responses (Hogg 1989).

Activated T cells are characterized by the expression of IL-2R (CD 2R and CD25) and a number of other newly expressed surface structures, the CD26 (dipeptidyl-peptidase IV), CD30, CD69, CDw70, CD71 (transferrin receptor). The insulin receptor is also absent on resting T cells and synthetized *de novo* by stimulated cells.

The surface of T cells possess a multitude of other receptors that interact with hormones, carrier proteins, pharmacoactive substances, neuropeptides and drugs. Immature T cells bear receptors for respective classes of thymic hormones.

We have shown that the AchE activity of peripheral blood lymphocytes is restricted to the T cells, especially to a low density fraction which represents an activated T cell population (Szelényi et al. 1982). In another series of experiments we have found a significant increase in acetylcholinesterase (AchE) activity and in muscarinic acethylcholin receptor (mAchR) expression in normal human peripheral blood lymphocytes as an early response to stimulation by mitogens, phorbol-myristylacetate (PMA), anti-CD3 mAb and the CA ionophor A23187.

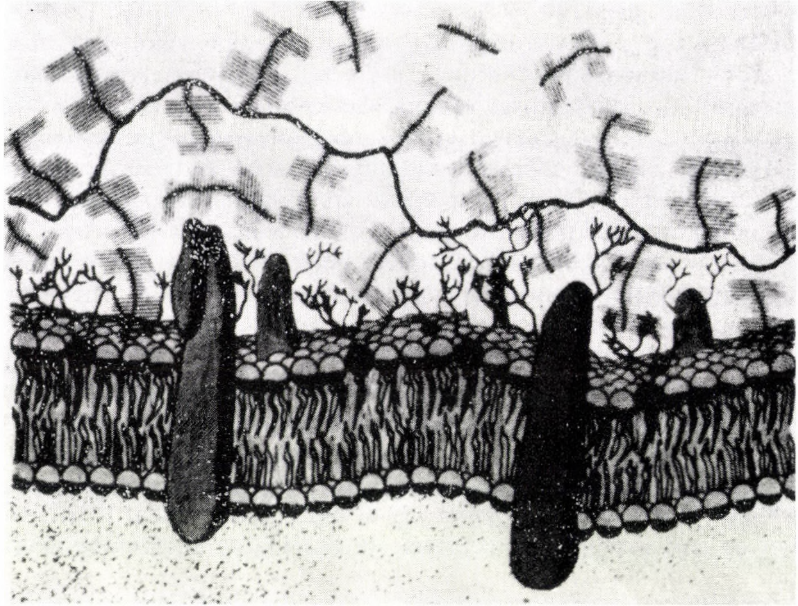

Fig. 10. The carbohydrate moieties of glycoproteins and glycolipids protrude into the extracellular space like antennas and interact with proteoglycans

In contrast to this, T-ALL cells showed a markedly decreased AchE activity, normal expression of mAchR, weak response to mitogens with no significant change in their initially higher cGMP values (Szelényi et al. 1987).

The Role of Carbohydrates

Studies with mAbs have shown that antigenic determinants of biologically important receptors and other surface molecules involved in cell to cell communication are carbohydrate antigens which, according to their carrier structures, are either glycoproteins or glycolipids (Fig. 10). Glycoproteins span the cell membrane. Glycolipids are much smaller and more cryptic. The sugar residues of both protrude like antennas from the external surface of the lipid bilayer and interact with the cloud of proteoglycans surrounding the cells. Biological role of carbohydrate groups received renewed attention, since the sequence diversity and the infinite variability of the carbohydrate code provides signals for protein targeting and cell-cell interactions. Carbohydrate recognition signals render the possibility for 'social' interactions of cells. These signals are the language by which cells can talk to each other. I have already mentioned that the tissue specific recognition of lymphocytes by endothelial cells is highly dependent on the specific

glycosylation of the cells. The anti-carbohydrate monoclonal antibody (HNK-1) originally raised against a human NK cell-line recognizes cell adhesion molecules distributed on both the nervous system and the immune system. These neural adhesion molecules seem to be important for the entry of lymphocytes into the brain. I would even surmise an eventual role of these structures in the development of 'sanctuaries' for malignant cells in the central nervous system. Dramatic changes of the oligosaccharide moiety of glycoconjugates are often associated with ontogenic and oncogenic events without affecting cell viability. In different stages of cell development, a repertoire of different glycoforms of proteins may have a unique spectrum of biological activities. Besides species specific and tissue specific glycoslyation, the glycotype of a cell is defined by its unique glycosylation capacity. This depends on differences in the level, activity and expression of individual intracellular glycosidases and glycosyl-transferases, as well as on intracellular routing, topography and exposure to these enzymes, availability of necessary metabolites and the activities of ecto- and extracellular enzymes. Tissues appear to create markedly few glycoforms in common.

The posttranslational glycosylation of polypeptides serves to generate discrete subsets of glycoproteins leading to finely tuned functional diversity. The glycotype defines the cellular identity. The recognition of this by other cells requires a complementary array of lectins. The carbohydrate moiety confers also important physical properties on the carrier proteins and lipids, such as conformational stability, protease resistance, charge and water binding capacity. Water molecules influence molecular interactions in all biological systems. Ordered water molecules can contribute directly to the properties of proteins by influencing their interactions with ligands.

Pitfalls of Inferences on Interacting Cell Surface Structures

Taking into account all aforementioned variability of factors influencing cell to cell interactions, we cannot wonder about a number of contradictions arising between the results of in vitro and in vivo investigations. Structural studies of the interaction in antigen-antibody complexes focus purely on spatial arrangement of atoms. The functional approach has to introduce, however, the fourth dimension, time. Methods, which operate within a functional perspective actually analyze cross-reactivity, since the hydrogen bonds at the epitope-paratope interface have dynamics of binding and breaking in the course of interactions. It is, however, a serious pitfall that experimental conditions of binding assays influence the conformation of the antigenic peptide. Assay conditions will, in fact, determine whether binding is observed or not, and thus whether certain residues are scored as belonging to the functional epitope (Regenmortel, 1989).

Anti-peptide antibodies are extremely useful for isolating and characterizing gene products. The high success rate in this case is partly due to the fact that cross-reactive antibodies need not necessarily be specific for the native confor-

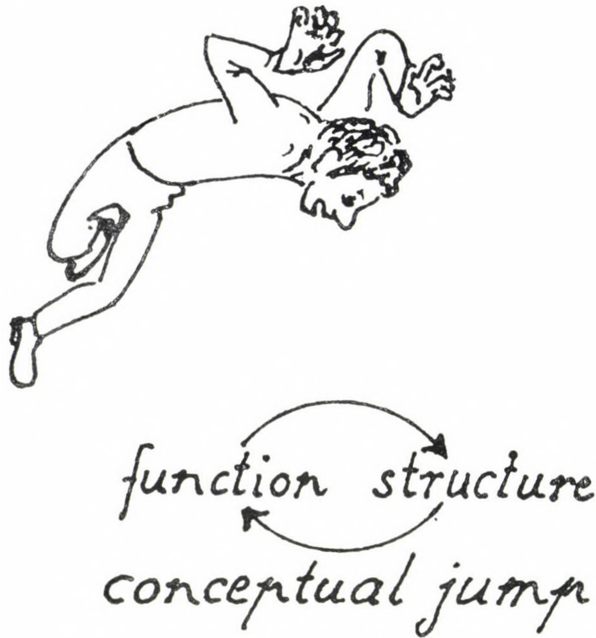

Fig. 11. Quite often a conceptual jump is needed

mation of the protein to detect the antigen in many of the currently used immunoassays. This does not diminish their practical value, but we simply have to keep in mind that in an attempt to understand the real structural basis of protein antigenicity in addition to the aminoacid sequence and the tertiary structure, accessibility, protrusion, mobility and hydrophilicity also have to be taken into account.

It is now generally recognized that the systems under investigations in biological sciences exhibit such a high degree of complexity that traditional notions of linear casuality do not apply. In discerning the highly important epitope-paratope interactions in cell to cell conversation, the more analytical framework of network casuality has to be applied, since a large number of interacting factors and variables *seem to influence* a particular feature of the system *instead of causing it*. Quite often a conceptual jump is needed to go from structure to function and vice-versa (Fig. 11). It is, however, extremely important to differentiate between observation and inference. It happens quite often that conclusions and categorizations based on inferences are being too quickly and uncritically applied in clinical practice.

We cannot dispense with the molecular structure and the physicochemistry of cell to cell interactions if we want to understand the real language of conversation between the cells of our organism.

Conclusion

1. Direct cell to cell contact is needed for conversation between T cells and antigen presenting cells:
- strong bonds are needed for stabilizing interactions between motile cells,
- for concentration of action to the area of cell to cell contact,
- for bidirectional exchange of crucial information via transient mobile synapses and
- for the regulation of lymphocyte traffic.

2. The recognition of the MHC presented peptides is conformational. Protrusions, accessibility, mobility and hydrophilicity of the interacting structures as well as the dynamics of bonding at the interface have to be taken into account.

Steric models of the TCR/antigen/MHC complex point to the antigen dependent molecular basis of MHC restriction.

3. A broad spectrum of T cell surface molecules modulate the immune response.

4. Carbohydrate recognition signals form the finely tuned language for "social interactions" between cells.

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Policies on Iron in Denmark

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The mounting evidence on the adverse effects of increasing iron stores raises the question of the appropriateness of existing national policies on iron fortification of foods and iron supplementation in the development countries. A pilot study on the pattern of iron supplementation during pregnancy was implemented in one county of Denmark. The results of the study suggest that there is inappropriate use of iron and laboratory investigation for haemoglobin during pregnancy. For recommendations to be made at national level similar studies should be performed in other counties of Denmark.

Keywords: iron, iron policies, iron supplementation, iron fortification

Iron deficiency anaemia (IDA) is basically a health problem associated with poverty and ignorance. The most vulnerable groups are pregnant women, infants and children, who all have increased demands for iron due to the rapid growth and the physiological changes they undergo.

Denmark, like most other countries in the European region, realising the magnitude of the problem of IDA and the serious consequences that it can have, introduced supplementation and fortification programmes. These were introduced in the post-war years, about 30–40 years ago, when IDA was a public health problem and when it was a cause of morbidity and mortality.

The situation in Denmark today is different. Socioeconomic conditions have improved tremendously, education level is high, eating habits and patterns have changed, food is abundantly available, vitamins and minerals are within easy reach of the people, and in general infections are well under control. All these factors have lead to the virtual disappearance of IDA, with the exception of a small percentage of women. There is, on the other hand, an increasing concern and interest on the hazards associated with increased iron intake and iron stores, and the judiciousness of continuing old and established supplementation and fortification programmes without proper objective assessment.

Haemochromatosis, which was previously considered a relatively rare condition, is now occuring more commonly in countries like Sweden, which has been one of the strong promoters and implementers of food fortification programmes over the last 30–40 years [1, 2]. Fortification programmes could have also

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contributed to accelerating the course of the disease in persons with preclinical haemochromatosis, thus causing them to present themselves with clinical signs and symptoms at an earlier age [3].

High haemoglobin levels and increased iron stores have also been regarded as "risk factors" for the development of myocardial infarction, which is one of the major killers in affluent societies; and that in fact, mild anaemia and lower iron stores protects one from heart disease [4, 5, 6].

High body iron stores have also been linked to increased risk of cancer [7]. The iron and protein, traditionally associated with the metabolism of iron, have shown to suppress immune function. Of these the most extensively documented is ferritin which has been shown to suppress T cell functions and to be associated with causing cancer [8]. Further, the role of iron and its importance in the etiology of rheumatoid disease has also been suggested [8, 9].

In the association of iron with infection, the critical question is not whether iron deficiency is associated with fewer infectious complications, but whether correcting the hypoferraemia of inflammation may deleteriously affect existing infection [10].

An average Danish diet today contains 10–20 mgs of iron per day, this is 10 times more than the amount required to replace the iron lost daily through the normal physiological processes [11]. It has been pointed out by the National Food Agency (Levnedsmiddelstyrelsen) that the low iron stores in the Danish women is not caused by low intake of iron in their diet, but by a low total intake of energy. Therefore, iron fortification of foods, if it is aimed to help alleviate or present low iron stores in these women, will not help them at all [12]. Recognising this fact, it has been decided that iron fortification is not an absolute necessity in a population that is not iron deficient. Denmark has, since early 1987, made iron fortification of flour and cereals voluntary, but none of the producers have wanted to fortify these foods. It is, however, statutory to fortify infant formulae with iron [13, 14].

As women during pregnancy are at higher risk of developing IDA, iron supplementation has been one of the chief activities of antenatal care, and iron supplements are prescribed widely, starting from the first trimester of pregnancy, without any regard to the individual partient's needs. This uncritical prescription, without taking into account that it produces unnecessary and unpleasant gastrointestinal side effects, is usually justified on the basis that dietary intake is precarious in relation to needs, the preparations used are without harm, and deficiencies are so common as to justify supplementing the whole population [15]. Routine iron supplementation, if recommended, can be delayed until after 28 weeks of pregnancy [16]. A review of 17 controlled clinical trials, on iron and vitamin supplementation during pregnancy in developed countries, found that there was little or no evidence of obvious benefit from iron to either the mother or the foetus [17]. Also, low iron stores do not have the same importance in pregnant women as in the non-gravidae, and in practice half have restored their iron stores within two months of delivery [15]. Further, maternal iron deficiency does not

lead to complications during delivery and does not cause neonatal iron deficiency [18, 19]. All this evidence questions the value of iron supplementation to those with mild and moderate IDA during pregnancy.

For a long time it was believed that higher maternal haemoglobin was an indicator of good prognosis or outcome for the pregnancy. But now, studies have found that high haemoglobin levels are associated with foetal disorders, both growth retardation and intra-uterine death of unknown cause, before the beginning of labour [20] and low birth weight [21]. Is it then justified for non-anaemic women to receive iron supplementation during pregnancy?

Blanket administration of iron in areas where there are no obvious indications has been questioned by the WHO. The WHO recommendation for iron supplementation during pregnancy is that it should be practised primarily in the second half of pregnancy when the iron requirement is greatest [22]. As the maintenance of haemoglobin concentrations at non-pregnant levels does not seem necessary, the need for prophylactic iron should then be reassessed in the light of improvements in the nations health, and newly applied strategies suited to the real needs of the people need to be developed. In Denmark the policy on iron supplementation during pregnancy has remained unchanged until now.

Target 38 of the Health for All by the Year 2000 policy states: "Before 1990, all Member States should have established a formal mechanism for the systemic assessment of the appropriate use of health technologies and of their effectiveness, efficacy, safety and acceptability, as well as reflecting national health policies and economic restraints." Realising that iron is not being given enough attention in the right perspective by health planners and policy makers, the Quality of Care and Technologies (QCT) programme of the World Health Organisation Regional Office for Europe, selected it as on of its key areas of activities.

In 1986, QCT, WHO in collaboration with the International Society of Haematology and the International Committee for Standardization in Haematology, held an International Workshop on Appropriate Iron stores in Pilisszentkereszt, Hungary [23]. During this workshop it became clear that the optimal iron state is still unknown and that extensive biochemical, clinical and epidemiological investigations are needed to determine where this optimum should be placed. On the specific area of supplementation and fortification, it was recognised that there was a need for the review of national policies since there is a wide variation between different countries. These expected variations could be helpful for targeting further research on health consequences and policy decisions.

This Workshop resulted in further studies that are in various stages of development. A study on iron supplementation during pregnancy – a randomized controlled trial, on a sample of 2912 women in Finland – has been completed, and preliminary results show that there is no benefit of iron supplementation during pregnancy (verbal communication). Another study which examines the effect of iron quantity in milk formulae on child growth and health in Hungary is still in progress.

A pilot study on the appropriate utilization of iron – through the review of national policies on supplementation and fortification – and the study of supplementation patterns during pregnancy in Storstroems County, Denmark is now nearing completion. Preliminary results show that three of the 185 pregnant women had haemoglobin levels below 11 gm% at the beginning of their pregnancy (between 8–12 weeks), 122 (66%) of them were started on iron supplementation during the first trimester. The prescription of iron appears to be unrelated to the haemoglobin levels. Six women decided not to take iron because they felt that they had no need for it. 49 of the 179 (27%) women who took iron supplementation suffered side-effects. A majority (75%) of them did not pay attention to their diet, and did not increase intake of foods rich in iron during their pregnancy. In Denmark, the National Board of Health is in the transition phase of revising its policy on iron supplementation during pregnancy. The new policy will recommend that supplementation be given only to those needing it. The earlier policy was that all women should receive iron supplementation from the first trimester. The results of the pilot study show that:

1. diet, which is the basic cause of IDA, needs to be given more attention during pregnancy,
2. the side-effects suffered could have been minimised if supplementation, in those not anaemic, had been postponed to the second trimester of pregnancy,
3. results of the haemoglobin tests were not influencing treatment patterns,
4. the practice of supplementation during pregnancy conforms with the earlier national policy, but not with the proposed one.

The above indicates that there may be inappropriate use of iron during pregnancy, and the laboratory investigations for the diagnosis of anaemia. This assumption, however, is based on the practice in one county only. For recommendations to be made at a national level, studies need to be done in other counties of Denmark as well. The experience gained may not only be of national value but may serve as a model for other countries in investigating and promoting the appropriate use of iron internationally.

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Fibronectin and the Adhesive Properties of Rat Lymphocytes Obtained from Different Peripheral Lymphoid Tissues

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A comparative investigation has been carried out on the effect of plasma fibronectin (Fn) on the adhesive properties of normal rat lymphocytes obtained from different lymphoid tissues: blood, spleen, mesenteric and tonsillar lymph nodes. Fn was immobilized on the basis of its ability to bind to gelatin. We established that concentrations of 40–50 μg/ml are sufficient for a saturation effect on Fn coating. For spleen cells an adhesion of 55.7 ± 9.3%, for mesenteric lymph nodes 34.5 ± 8.7% and for tonsillar cells 33.8 ± 3.2% was observed. Blood lymphocytes showed the lowest adhesion, 21.3 ± 4.2%. Compared to the other lymphoid tissues, the spleen cells exhibited a "basal" adherence to surfaces coated with gelatine only: 19.2 ± 4.1%. T lymphocytes participate to a greater extent in the process, since their number was significantly reduced in cell suspensions after adhesion to both gelatin and gelatin-Fn coated surfaces. The addition of soluble Fn leads to a competitive inhibition of the lymphocyte adhesion to gelatin-Fn coated surfaces.

The data demonstrated the important role of Fn for the adhesive interactions of lymphocytes during their functional distribution in the tissues.

Keywords: lymphocyte adhesion, fibronectin, lymphoid tissues

Introduction

Fibronectin (Fn) is a major component of the extracellular matrix (ECM). It is known to promote cell adhesion and to affect growth and differentiation of various cells [1]. The mechanism by which lymphoid cells recognize and adhere to Fn or to other components of ECM has been intensively investigated during the past few years [2, 3]. Recent studies [4, 5] showed that these cells have adhesive receptors, which can mediate cell-to-cell or cell-to-matrix interactions. They seem to play an important role in the migration of lymphocytes into extravascular tissues, in complex formation with antigenic target cells and in the recirculation to lymphoid organs [4]. The results published in our previous communication suggest the presence of a lymphocyte subpopulation in human peripheral blood, showing an ability to interact with both immobilized and soluble Fn [8].

In the present study we continued our experiments in a rat's lymphocyte system, making a comparative investigation on the effect of plasma Fn on the

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adhesive properties of lymphocytes obtained from different peripheral lymphoid tissues, such as spleen, mesenteric and tonsillar lymph nodes and blood. This was done to answer the question whether the adhesive behaviour of lymphocytes, especially the interaction with Fn, is connected with their functional distribution and migration in the organism.

Materials and Methods

Preparation of lymphocyte suspensions

Rat lymphocytes were obtained from various peripheral lymphoid organs according to Hudson and Hay [8]. Sodium citrate (3.8%) in 9 : 1 ratio was used as an anticoagulant. The blood was drawn from v. cava inferior and after the animals were killed the spleen, mesenteric and tonsillar lymph nodes were removed. Single cell suspensions were made by gentle stimulation of the organs with a needle, washing with PBS and filtering through a nylon mesh. The cells were further purified by differential gradient centrifugation on Lymphoprep (Niegaard & Co. A/S Oslo, Norway, r.w. 1,077) and resuspended in Eagle medium to a final concentration of 5×10^6 cells/ml. The purity of the lymphocyte suspensions was higher than 95%, contaminated with a small amount of red blood cells (1–3%) and macrophages (2–4%). Viability was determined by trypan blue exclusion test and was higher than 90%.

In some experiments we measured the amount of T lymphocytes with T-rosette determination, using fresh rabbit erythrocytes, according to Taswell [9].

Determination of the lymphocyte adhesion

The adhesion assay was carried out in 96 ELISA plates with flat bottomed wells (Type Novogen, Czechoslovakia) as previously described [7]. Briefly, 5×10^6 cells in 0.1 ml medium were pipetted into each well and the plates were incubated at 37 °C for 60 min in a humidified 5% CO_2 air incubator. At the end of the incubation, the nonadherent cells were removed by washing twice with PBS. The cells that adhered to the bottom of the wells were fixed with 2.5% glutaraldehyde for 15 min, stained with 1% methylene blue in PBS for 1 hour, and washed twice again. In some cases, the supernatant from each well was resuspended gently and collected in order to estimate the concentration of unattached cells.

The degree of adherence was assayed in two ways: (a) by counting the unattached cells and calculating the percentage of adhesion, and (b) photometrically, by measuring the optical density of the dye bound to the attached cells. In the assay (a) the supernatant from each well was gently collected and counted

in a haemocytometer. The percentage of adhesion was calculated by the following formula:

$$\text{Adhesion (\%)} = \frac{\text{Number of viable cells after incubation}}{\text{Number of viable cells before incubation}} \times 100$$

In assay (b), after staining the attached cells (see above), 100 μl of 70% ethanol was pipetted into each well and absorbance was estimated after 30 min at 650 nm in an ELISA processor (Sumal, Zeiss, DDR). The results obtained using method (b) showed a satisfactory correlation when compared to method (a).

Purification of fibronectin

Acid citrate dextrose plasma (ACD-plasma) was collected from healthy donors. The fibronectin was prepared by affinity chromatography on gelatin-Sepharose 4B according to the method of Engval and Ruoslahty [10] and stored in 4 M urea. Before utilization the Fn was transferred to 120 mM NaCl and 50 mM Tris, pH 7.3 by gel filtration with Sephadex G-25. The purity of the Fn was assayed by SDS polyacrilamide electrophoresis (7.5% slab gel). A single band was observed at 220 kDa.

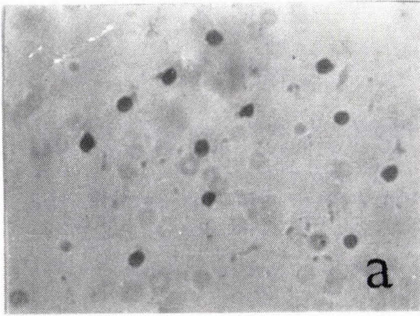
Preparation of gelatin and gelatin-fibronectin coated surface

The gelatin coated surface were prepared by addition of 100 μl gelatin (Sigma, 300 bloom) solution at a concentration of 50 μg/ml in PBS to wells, incubating the plates overnight in a humidified thermostat at 37 °C, and subsequently washing them three times with PBS. Immobilization of Fn was carried out on the basis of its ability to bind to gelatin. For this purpose some of the gelatin coated wells were additionally exposed to various concentrations of Fn (2.5 – 80.0 μg/ml) in PBS for 30 min at room temperature.

Results

The adhesion of rat lymphocytes to Fn coated surface was investigated. The target of this study were normal lymphocytes isolated from blood, spleen, mesenteric and tonsillar lymph nodes. The ability of Fn to interact with denatured collagen (i.e. gelatin) [10] was used in order to model a binding of this protein as similarly as possible to physiological conditions.

Typical experimental data concerning the adherence of different lymphocytes on gelatin coated and gelatin – Fn coated wells are shown in Fig. 1. A marked increase is apparent in the lymphocyte adherence to Fn coated wells. Counting the concentration of unattached cells, the following percentages of adhesion were found: spleen cells 55.7 ± 9.3%, mesenteric cells 34.5 ± 8.7%, tonsillar lymph node cells 33.8 ± 3.2%, blood lymphocytes 21.3 ± 4.2% (see Table 1).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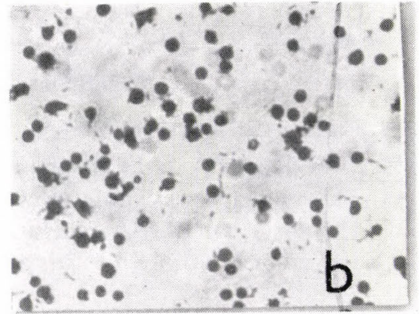
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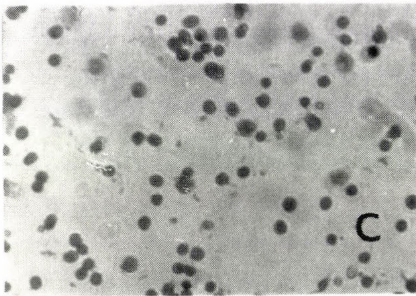
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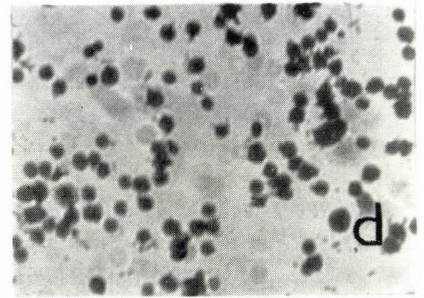
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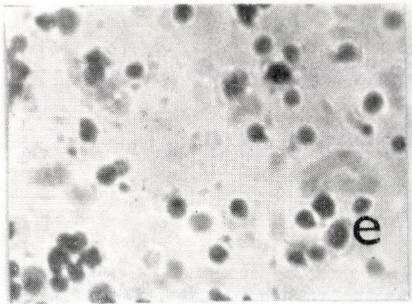
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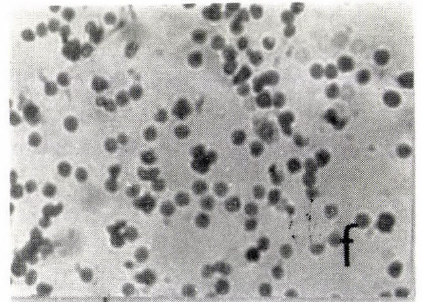
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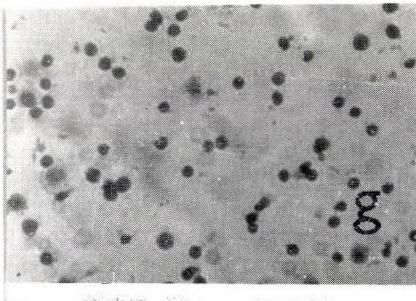
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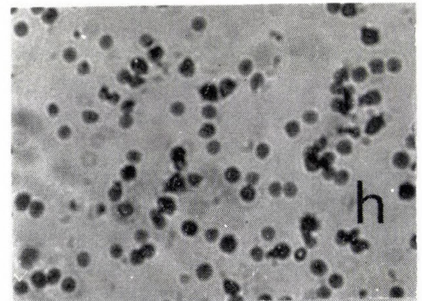
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Table 1

Adhesion of rat lymphocytes obtained from different peripheral lymphoid tissues to gelatin and gelatin-fibronectin coated surfaces

Lymphocyte source	Adhesion in % (mean + S.E.M.)	
	gelatin coated alone	gelatin-Fn coated
Blood	11.2+0.7	21.3+4.2
Spleen	19.2+4.1	55.7+0.3
Mesenter. lymph nodes	12.0+1.0	34.5+8.7
Tonsillar lymph node	14.7+0.9	33.8+3.2

A certain augmentation in the basal adherence to wells coated with gelatin only was observed for spleen cells (19.2 ± 4.1%), when compared to the other lymphoid tissues (Table 1).

The curves for the lymphocyte adhesion induction (measured photometrically) as a function of the coating concentration of Fn are shown in Fig. 2. It is evident that concentrations of 40–50 μg/ml are necessary for achievement of maximal effect of Fn coating. Independently of the quantitative differences, the curves for lymphocytes from various tissues were similar and a plateau was reached at almost equal concentrations of Fn.

In Fig. 3, the adhesion to Fn is presented as Adherent Index (AI) (indicating the mean ratio between adhesion to Fn and corresponding gelatin (control) surface. The results from 11 separate experiments (using different animals) showed that the spleen lymphocyte population exhibits a maximal ability to interact with Fn, where $AI = 2.7 + 0.2$ ($p < 0.05$), followed by lymphocytes from mesenteric and tonsillar lymph nodes (which were not distinguishable from each other) $AI = 2.4 + 0.1$ and $2.5 + 0.2$, respectively ($p > 0.05$). The lowest AI is characteristic of blood lymphocytes – $2.0 + 0.1$ ($p < 0.05$).

In this system, the effect of soluble Fn was tested, since this protein is a normal component of the blood plasma (as well as other biological fluids) and could compete with immobilized Fn in cell receptor binding [7]. A significant

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Fig. 1. Morphology of different rat lymphocytes adhered to gelatin coated (a, c, e, g) and gelatin-Fn coated wells (b, d, f, h). (a, b)-blood lymphocytes; (c, d)-spleen; (e, f)-mesenteric lymph node; (g, f)-tonsillar lymph node. Rat lymphocytes were obtained from different peripheral lymphoid organs (see Materials and Methods) and were incubated for 1 hour at 37 °C in polystyrol plates precoated with gelatin or gelatin-Fn (50 μg per ml for 30 min). At the end of incubation nonadherent cells were removed by washing twice with PBS. The cells adhered to the bottom of the wells were fixed with 2.5% glutaraldehyde and stained with 1% methylene blue

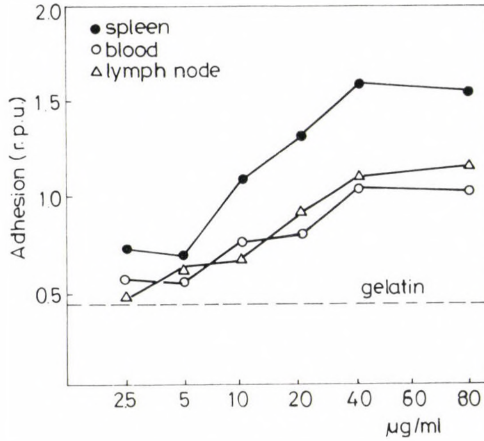

Fig. 2. Lymphocyte adhesion induction as a function of the coating concentration of Fn. Different concentrations of Fn in 50 mM this 120 mM NaCl, pH 7.4, were used for a second coating of gelatin precoated wells. The degree of adherence was assayed photometrically (r.p.m.-relative photometrical units) as described under Material and Methods. Each point is the mean of four experiments

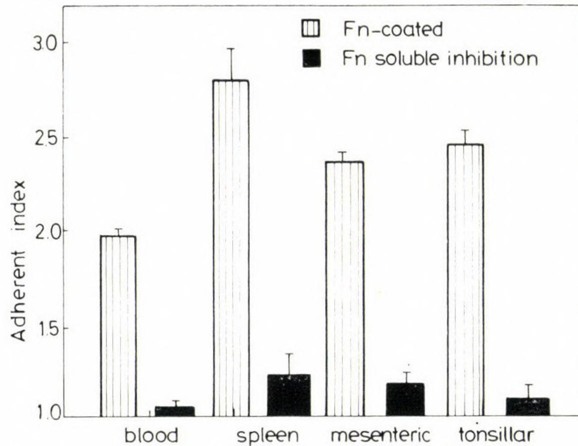

Fig. 3. Effect of Fn on the adhesive properties of lymphocytes from different peripheral lymphoid tissues. The adhesion to immobilized Fn is presented as Adherent index (mean ratio between lymphocyte adhesion to gelatin-Fn and gelatin coated wells). The inhibitory effect of soluble Fn (in concentration 200 µg/ml) on the lymphocyte adhesion to Fn coated surface (gelatin-Fn wells) was also tested. The degree of adherence was assayed photometrically (see Material and Methods). Each value is mean $\pm$ Confidence Limit ($p = 0.05$) from 11 different experiments. The measurements were performed in triplicate

Table 2

T (B) lymphocyte content

Lymphocyte source	Before adhesion		After adhesion			
			gelatin		gelatin-Fn	
	T (%)	non T (B) (%)	T (%)	non T (B) (%)	T (%)	non T (B) (%)
Blood	56	44	39	61	35	65
Spleen	51	49	37	63	25	75
Mesenter. lymph node	45	55	23	77	31	69
Tonsillar lymph node	58	42	45	55	43	57

decrease of lymphocyte adhesion to gelatin-Fn coated surfaces was observed and this effect was similar for cells prepared from various lymphoid tissues (Fig. 3).

In some experiments, the amount of T lymphocytes in the suspension (respectively non T (B) lymphocytes) before and after adhesion was evaluated, in order to find out which cells are involved in the adhesive process. The data are presented in Table 2. A definite tendency for a reduction of T cells after adhesion to both gelatin and gelatin-Fn coated surfaces was measured.

Discussion

Our data indicate that there exists a subpopulation of lymphocytes, which is able to interact with Fn. This fact is in good accordance with our previous study [8], and also some recent investigations concerning the human lymphocytes [4, 5, 6, 7].

In the present work we showed that there are significant differences in the lymphocyte adhesion to Fn, depending on the lymphoid tissues they originate from. The highest adhesion was exhibited by spleen lymphocytes where more than 50% of the cells adhered, followed by lymphocytes from tonsillar and abdominal pools (35% and 33%, respectively). The lowest adhesivity was shown by blood lymphocytes (approx. 21%). It should be noted that the values observed for blood lymphocyte adhesion are in good accordance with our previous data concerning the effect of Fn on human peripheral lymphocytes [7]. It seems that T lymphocytes participate to a greater extent, since their number was reduced by about 15–20% after adhesion. This, however, is valid for both lymphocyte adhesion to gelatin and gelatin-Fn coated surfaces. Kurki et al. [4] reported that mitogen stimulation promotes human T lymphocyte adhesion to Fn, indicat-

ing that the attachment of lymphocytes depends also on their functional state. However, not only the T cells are sensitive towards Fn. Liao et al. [5] showed that some B lymphoid cell lines adhere predominantly to Fn. We suggest that both T and B lymphocytes participate in adhesion (probably to a different degree). Otherwise, it is hard to explain the significant difference in adhesion, for example between spleen and blood lymphocytes, in spite of the fact that T cell diminution (Table 1) was similar. Obviously a more universal mechanism of attachment to Fn, which is not related to their subpopulation characteristics, might be valid in the case. In this respect, the differences in the lymphocyte adhesion to Fn which we observed are probably a result of the accumulation of these cells in organs, where lymphocytes exist mainly in an immobilized state. It is difficult to explain, however, why of all lymphoid cells spleen lymphocytes showed the highest adhesiveness to Fn. One possibility is that it may be due to the functional storage of a certain part of "adhesive" blood cells (including lymphocytes) in sinusoidal vessels [11].

In a previous paper we reported the inhibition of lymphocyte adhesion on Fn coated surfaces in the presence of soluble Fn in the medium [7]. This effect was confirmed in the present experiments, indicating that the lymphocytes could apparently interact with both immobilized and soluble Fn. According to our concept, the interaction of Fn receptor with solid phase-immobilized Fn leads to cell adhesion, while preliminary saturation of the receptor with soluble Fn induces a competitive inhibition of the process. It is noteworthy, however, that this is not typical of other cell systems, for example in fibroblast or hepatocyte adhesion [5]. This suggests the existence of some differences between the Fn receptor in lymphocytes and in other cells. A specific 140 KDa receptor for Fn was recently isolated from the MOPS 315 lymphoid cell line, which exhibits a cross immunoprecipitation with the BHK fibroblast Fn receptor [5]. A similar, but 190 KDa Fn receptor protein was also isolated from activated T lymphocytes [4]. The problem of the type of interaction of the soluble Fn and the corresponding lymphocyte membrane receptor (which occurs for example in blood or other biological fluids) also remains unsolved. Most likely this interaction is comparatively weak, since Fn could be washed away in the course of lymphocyte preparation. Otherwise it would not be possible to monitor any effect of Fn in these experiments.

In the present work we also established certain differences in the adhesion of lymphocytes on denatured collagen (i.e. gelatin). In general, the spleen cells exhibited a definitely higher tendency for attachment on this material, whereas blood or lymph node lymphocytes did not show any significant differences. Such results could be explained by the existence of some bound Fn on the lymphocyte surface, which could not be washed off during cell preparation, and which interacts with gelatin. Nevertheless, the direct interaction of lymphocytes with collagen (gelatin) should not be ruled out, since there is certain evidence in literature [6, 12] for the existence of a collagen binding protein (VLA 2), which is a component of the integrin complex [13].

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Interaction of Thrombin with Endothelial Cells in the Presence of Fibrinogen and α_2-Macroglobulin

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Binding of thrombin to cultured endothelial cells has been studied in the presence of fibrinogen and α_2-macroglobulin. Both fibrinogen and α_2-macroglobulin inhibit the interaction of thrombin with endothelial cells. Whereas fibrinogen decreases the rate of activation by the thrombin-thrombomodulin complex of protein C, thrombomodulin inhibits the rate of inactivation by α_2-macroglobulin thrombin. α_2-macroglobulin also binds to endothelial cells; ($K_d = 3 \times 10^{-7}$ M with 3×10^5 binding sites/cell), and the rate of binding of the α_2- macroglobulin to endothelial cells is faster than its complex formation with the thrombin. The data suggest that essentially the cell-bound form of fibrinogen and α_2-macroglobulin influences thrombin binding and functions.

Keywords: aortic endothelial cells, fibrinogen, α_2-macroglobulin, protein C, thrombin, thrombomodulin

Introduction

Thrombin interacts with both molecular and cellular components of hemostasis [1, and references therein]. Its function is highly controlled by endothelial cells [2, and references therein]. The in vivo interaction of thrombin with endothelial cells occurs in the presence of high concentration of plasma proteinase inhibitors and fibrinogen. These proteins, besides being substrates of thrombin, can also bind to endothelial cells directly, with different affinities and at various rates [3–6]. The interaction of endothelial cell with thrombin may be modified by either free or cell-bound form of these plasma components.

In the present work, we examined the effect of fibrinogen and α_2-macroglobulin on the binding of thrombin to endothelial cells. We have found that α_2-macroglobulin binds rapidly to endothelial cells. The cell-bound form of the inhibitor or fibrinogen interferes primarily with the binding of thrombin to endothelial cells.

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Materials and Methods

Sepharose 4B, Sephadex G-25 and Sulphopropyl Sephadex C-50 Sephacryl S-300 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Fibrinogen (human, grade L) and the chromogenic peptide substrate, H-D-Phe-Pip-Arg-pNA $\times$2HCl (S-2238) were the products of KABI AB and KABI Diagnostica, Stockholm, Sweden, respectively. ^{125}I (as NaI, tracer free) was purchased from the Isotope Institute of the Hungarian Academy of Sciences, Budapest, Hungary. Lactoperoxidase (B grade, 42 I.U./mg) was from Calbiochem. Other chemicals were obtained from the Factory of Laboratory Chemicals (Reanal) Budapest, Hungary.

Human α-thrombin was prepared as published earlier [7, 8]. Protein C was prepared from human plasma [9]. Thrombomodulin was isolated from rabbit lung according to the method of Esmon et al. [10]. Human α_2-macroglobulin was prepared as described earlier [11, 12]. Antithrombin III was isolated from human plasma [13].

For the activation of protein C, thrombin and thrombomodulin was incubated for 5 min at 37 °C, thereafter protein C was activated with the thrombin-thrombomodulin complex (25 nM) for 10 min at 37 °C in 20 mM TRIS HCl 0.1 M NaCl buffer, pH 7.4, containing 10 mM $CaCl_2$ as described in [14]. Thrombin activity was neutralised by the addition of antithrombin III (2 μM) and heparin (0.5 mU/ml). Neither the thrombomodulin nor antithrombin III inhibited the amydolytic activity of the activated protein C on S-2238 substrate.

The activity of thrombin was blocked by a 100-fold molar excess of di-isopropyl fluorophosphate (DFP) at 4 °C for 1 hour. Excess amount of DFP was removed by gel filtration on a Sephadex G-25 column. Chemical modification of lysine side chains of fibrinogen was carried out with pyridoxal 5'-phosphate (PLP) as described in [5]. PLP and $NaBH_4$ were used in a 100-fold molar excess.

Protein concentrations were determined by the method of Lowry et al. [15]. Molar concentrations were calculated using molecular masses 36 KD; 720 KD; 62 KD; 75 KD; and 65 KD for thrombin [7], α_2-macroglobulin [11], protein C [9], thrombomodulin [10] and antithrombin III [13], respectively.

SDS gel electrophoresis was carried out in 7% polyacrylamide slabs [16]. Proteins were iodinated by lactoperoxidase [17].

To prepare thrombin-α_2-macroglobulin complex, 2.8 μm ^{125}I-thrombin and 2.8 μM α_2-macroglobulin were incubated at 22 °C for various times. Thereafter ^{125}I-thrombin and ^{125}I-thrombin-α_2-macroglobulin complex was separated on Sephacryl S-300 column (0.8 $\times$ 5 cm), equilibrated with 0.1 M phosphate buffer pH 7.4.

Bovine aortic endothelial cell (BAEC) were obtained as described previously [18]. The vials contained approximately 2×10^5 BAECs. For binding studies they were washed three times with phosphate buffered saline, pH 7.4 containing 1 mg/ml bovine serum albumin. The ^{125}I-labelled ligands were added in 0.5 ml serum deficient Dulbecco's medium containing 2 mg/ml albumin, 10

mM HEPES, pH 7.4, and incubated. After incubation the cells were washed three times within 15 sec with PBS-albumin, and finally 5 ml of liquid-scintillation cocktail (Triton X-100-toluene-PPO-POPOP) was added for determination of radioactivity bound to the cells. For dissociation experiments, cells were incubated in the presence of ^{125}I-ligands as described above, and thereafter they were washed three times with fresh medium used for binding experiments. Finally, 0.5 ml of this medium was added. At various times the medium was changed and the radioactivity of the medium and of the cell fraction was determined.

Results and Discussion

The interaction and the functions of thrombin with and on the endothelial cells can be influenced by various factors. Among them, a possible role of fibrinogen and α_2-macroglobulin has been examined. Both proteins interact directly with thrombin and also with endothelial cells [6]. Thus, the question arose how fibrinogen and α_2-macroglobulin can affect the binding of thrombin to endothelial cells.

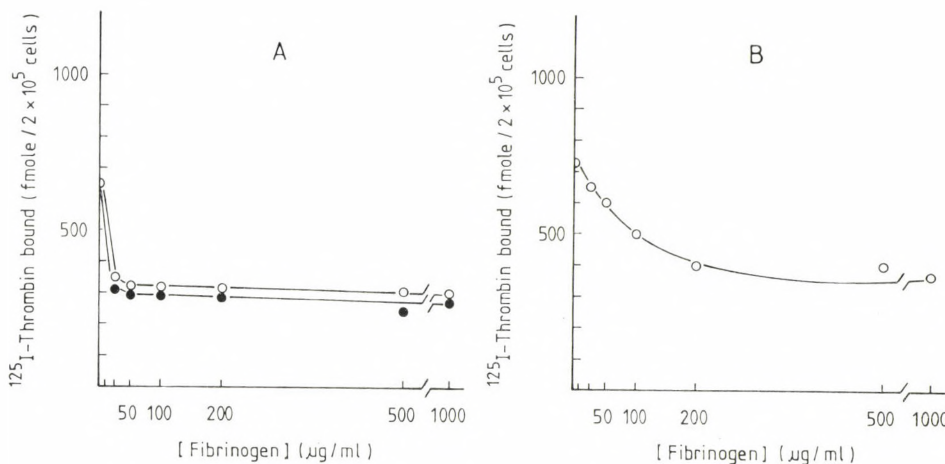

Fig. 1. Effect of fibrinogen on the interaction between thrombin and endothelial cells.

A. Binding of thrombin to endothelial cells in the presence of fibrinogen.
Endothelial cells (2×10^5 cells per vial) were preincubated with various concentrations of fibrinogen at 22 °C for 20 min. Thereafter, free fibrinogen was removed by washing and the cells were further incubated for 10 min in the presence of either 100 nM ^{125}I-thrombin-DIP (o) or 100 nM ^{125}I-thrombin-native (•). The amount of radioactivity bound to cells was determined.

B. Binding of active center blocked thrombin to endothelial cells in the presence of fibrinogen.
100 nM ^{125}I-thrombin-DIP was incubated with 2× 10^5 endothelial cells in the presence of indicated concentrations of fibrinogen. After 10 min incubation at 22 °C, the cell bound radioactivity was determined.

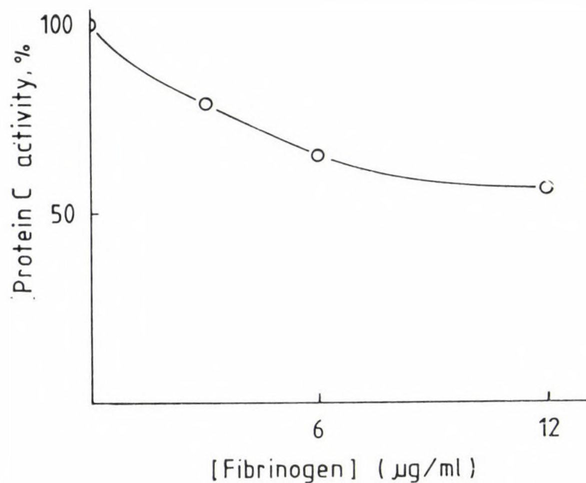

Fig. 2. Effect of fibrinogen on the rate of activation of protein C by thrombin-thrombomodulin complex.
Protein C was activated by thrombin-thrombomodulin complex in the absence or presence of various concentrations of PLP modified fibrinogen at 37 °C for 10 min as described in the Materials and Methods section. 100% activity represents the activity of activated protein C formed without the addition of PLP-fibrinogen

The binding of both native and DIP-thrombin was decreased by the preincubation of endothelial cells with fibrinogen (Fig. 1a). Similar results were obtained, when endothelial cells were incubated in the presence of ^{125}-I-DIP-thrombin and various concentrations of fibrinogen (Fig. 1b). These data indicate that the cell-bound fibrinogen cannot serve as an alternative binding site of endothelial cells for thrombin; it is rather inhibitory.

On the other hand, the cell-bound fibrinogen may modify some functions of thrombin on the cell surface. As it can be seen from Fig. 2, fibrinogen, modified by pyridoxal 5'-phosphate (PLP) to prevent clot formation, decreased the rate of activation by thrombin/thrombomodulin complex of protein C in a concentration dependent manner. Control experiments show that fibrinogen did not inhibit the activity of activated protein C on the synthetic peptide substrate S-2238 (not shown). All these effects of fibrinogen seem to provoke thrombus formation.

α_2-macroglobulin may also contribute to the regulation of thrombin activity. This inhibitor forms a complex with thrombin, in which the enzyme loses its capacity to clot fibrinogen. Furthermore, some data [19] suggest that α_2-macroglobulin interacts directly with endothelial cells, although no quantitative data are available in the literature. Therefore first we determined the kinetical parameters for the binding of α_2-macroglobulin to these cells. Our results show that α_2-macroglobulin binds to endothelial cells in a time and concentration dependent manner (Fig. 3 and Table 1). To attain equilibrium between free and cell-bound

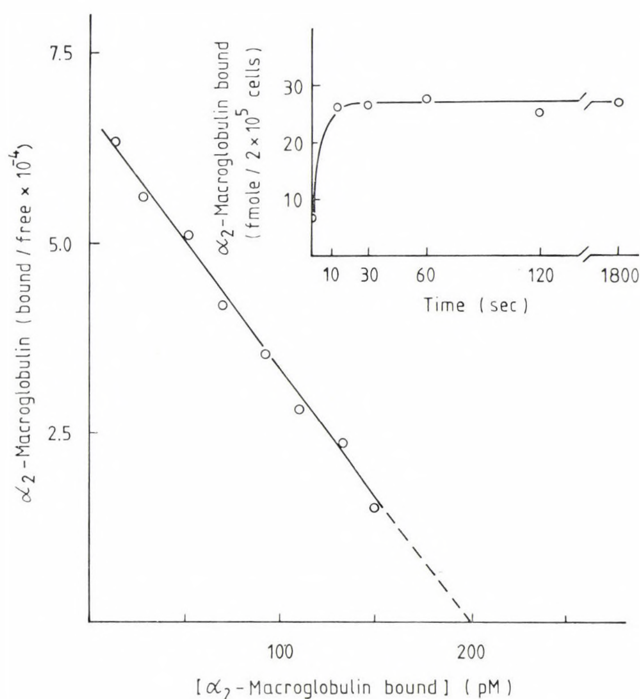

Fig. 3. The binding of α-$_2$macroglobulin to endothelial cells.
Endothelial cells (2×10^5 cells per vial) were incubated for 2 min in the presence of various concentrations of ^{125}I-α_2-macroglobulin (between 10 and 200 nM) at 22 °C. The ratio of bound (B) and free (F) α_2-macroglobulin is plotted as a function of bound inhibitor.
In inset: Time dependence of binding of α-$_2$-macroglobulin to endothelial cells at an inhibitor concentration of 50 nM

α_2-macroglobulin about 30 seconds are necessary. A 10-fold molar excess of unlabelled α_2-macroglobulin resulted in a 30% decrease in the binding of ^{125}I-labelled α_2-macroglobulin (data not shown). After the binding of α_2-macroglobulin

Table 1

Binding parameters for thrombin and α_2-macroglobulin

	K_d	Binding sites/cell
Thrombin$^+$	3×10^{-8} M	3×10^4
α_2-macroglobulin	3×10^{-7} M	3×10^5

$^+$ Data are taken from Ref. 5. Binding parameters for α_2-macroglobulin are calculated from Fig. 3.

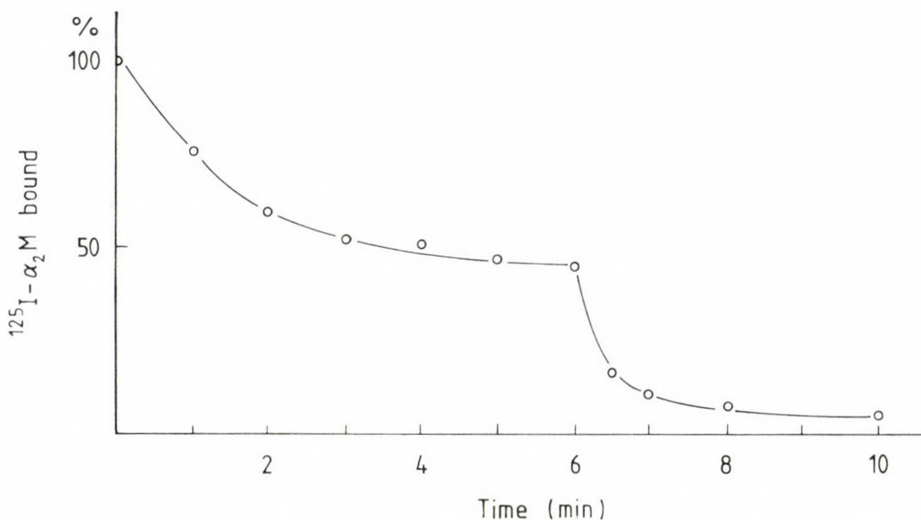

Fig. 4. Dissociaton of α_2-macroglobulin from endothelial cells.
Endothelial cells (2×10^5 cells per vial) were preincubated with 100 nM ^{125}I-α_2-macroglobulin for 2 min at 22 °C. Thereafter, free ligands were removed by washing, and the cells were further incubated in fresh media as indicated in Figure. After 6 min incubation, endothelial cells were treated with trypsin (100 μg/ml) for 8 min, and the cell-bound radioactivity was determined. Radioactivity bound to cells is expressed as a percent of the amount of α_2-macroglobulin bound at the end of the 2 min preincubation period

to endothelial cells a rapid dissociation occurred; 50% of the bound inhibitor was released in 3 min (Fig. 4). The remaining 50% radioactivity bound to the cells was releasable by trypsin treatment, indicating that α_2-macroglobulin is not taken up by endothelial cells at 22 °C. Since the concentration of α_2-macroglobulin in the circulation is high (about 3.5 μM), our results suggest that the endothelial cells are saturated with α_2-macroglobulin in vivo.

To study how α_2-macroglobulin influences the interaction of thrombin with endothelial cells, the cells were preincubated with the inhibitor and, thereafter the binding of ^{125}I-thrombin was determined. α_2-macroglobulin decreased the binding of thrombin to endothelial cells; a 20-fold molar excess of the inhibitor resulted in 36% decrease of binding of thrombin to endothelial cells (Table 2). Since thrombin could form a complex with α_2-macroglobulin, the binding of the inhibitor-enzyme complex to the endothelial cells was also studied. ^{125}I-thrombin was preincubated with molar excess of α_2-macroglobulin for 30 min. At various periods samples were taken and used for binding experiments. After 30 min incubation, the reaction mixture was applied to a column to isolate ^{125}I-thrombin-α_2-macroglobulin complex. The binding of the separated complex to endothelial cells was also determined. The results show the more complex formed the less radioactivity was bound to the endothelial cells (Fig. 5 and Table 2). The amount of ^{125}I-thrombin-α_2-macroglobulin complex bound to the endothelial cells was

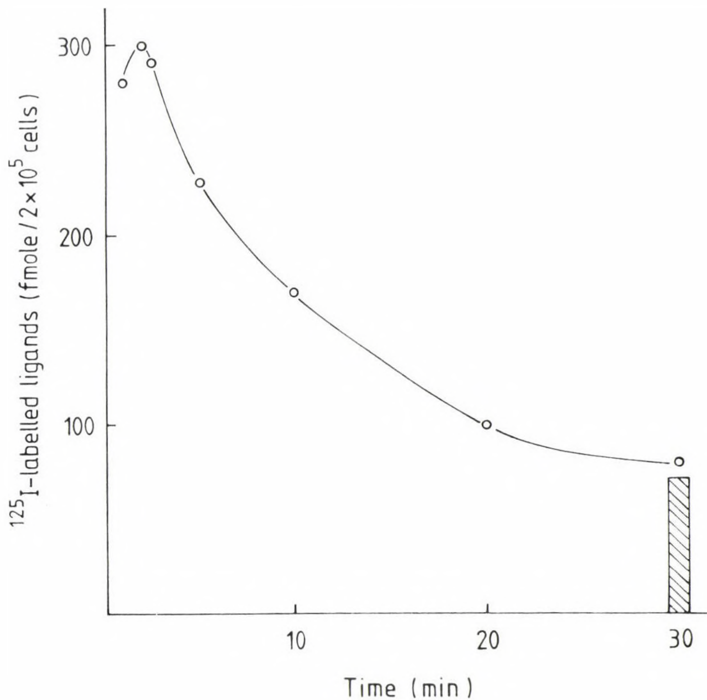

Fig. 5. Effect of α_2-macroglobulin on the binding of thrombin to endothelial cells. For direct binding studies ^{125}I-thrombin (100 nM) was preincubated with α_2-macroglobulin (125 nM) at 22 °C for 30 min. At various periods, as indicated, aliquots of reaction mixture were taken and added to endothelial cells. After 30 min incubation, the reaction mixture was gel-filtered as described in Materials and Methods, and the binding of the separated thrombin-α_2-macroglobulin complex to endothelial cells was also determined. The amount of radioactive ligands bound to endothelial cells (2×10^4 cells per vial) in a 5 min incubation period was determined. Binding of separated thrombin-α_2-macroglobulin complex is shown by the column

Table 2

Binding of thrombin to endothelial cells in the presence of α-macroglobulin

Concentration of α-macroglobulin		
none	250 nM	1000 nM
100%	84%	64%

Endothelial cells (2×10^5 cells per vial) were preincubated with indicated concentrations of α_2-macroglobulin at 22 °C for 1 min. Thereafter, 50 nM ^{125}I-thrombin was added and the reaction mixture further incubated for 1 min. The amount of cell-bound enzyme was determined as detailed in Materials and Methods. Results are expressed as percent of the thrombin bound without the addition of α_2-macroglobulin.

Table 3

Formation of thrombin-α_2-macroglobulin complex in the presence of thrombomodulin

	Time of the incubation (min)					
	1	2	3	5	10	20
Absence of thrombomodulin	10	15	20	40	50	70
Presence of thrombomodulin	9	10	10	15	15	20

^{125}I-thrombin (0.38 μM) was preincubated either in the absence or in the presence of thrombomodulin (2.1 μM) for 1 min. Thereafter α_2-macroglobulin 0.93 μM) was added and further incubated at 22 °C for times indicated in Table 3. Samples of reaction mixture were taken, applied to SDS gel electrophoresis for determination of the free enzyme and the enzyme-inhibitor complex formed. The gels were sliced and measured for radioactivity. Results are expressed as the percent of radioactivity in the thrombin-α_2-macroglobulin complex related to the total radioactivity (free thrombin plus thrombin-α_2-macroglobulin complex).

less than the amount of bound radioactivity of the samples, containing both ^{125}I-thrombin and ^{125}I-thrombin-α_2-macroglobulin complex. The data of Fig. 5 suggest, that the affinity of the inhibitor-enzyme complex to endothelial cells is less than that of the free enzyme. From these results we conclude, that the inhibitory effect of α_2-macroglobulin on the thrombin-endothelium interaction is the consequence of a complex formation between enzyme and inhibitor and it cannot be excluded that there is a competition between α_2-macroglobulin and thrombin for their binding to endothelial cells. However, observation concerning the effect of thrombomodulin on the α_2-macroglobulin thrombin complex formation (see later) in cell free system do not seem to support this assumption.

Since the rate of binding of both thrombin and α_2-macroglobulin is very rapid [5] (Fig. 3) while the reaction between thrombin and the inhibitor is slow (Table 3), we assume that the free α_2-macroglobulin may not play important role in the control of thrombin action on endothelium.

The possible influence of thrombomodulin on complex formation between thrombin and α_2-macroglobulin was also determined. ^{125}I-thrombin was incubated with α_2-macroglobulin either in the absence or in the presence of molar excess of thrombomodulin, and the amount of inhibitor-enzyme complex formed was determined by SDS gel electrophoresis. As shown in Table 3, thrombomodulin inhibits the reaction between thrombin and α_2-macroglobulin, similarly as heparin [20]. These data suggest that, the endothelium-bound thrombin can be quasi protected against α_2-macroglobulin.

Acknowledgements

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The Effect of Gamma-hexachlorocyclohexane (Lindane) on Blood Cells, Kidney and Liver Tissues in Rabbits

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The effect of intragastric administration of gamma-hexachlorocyclohexane (lindane) on peripheral blood cells, kindey and liver was studied in rabbits. White blood cells were found to be most affected by lindane, as was shown by the reduced phagocytic activity of neutrophils and the increased number of lymphocytes with inactive nucleoli.

The changes in the characteristics of erythrocyte cell membranes dependend on the length of time lindane was administered and on its withdrawal. Histological examination of the liver and kidney showed focal degeneration of tissue and cell structure.

Keywords: gamma-hexachlorocyclohexane, leucocytes, erythrocytes, kidney' liver

Introduction

Lindane is a hematotoxic compound that

- acts directly on bone marrow stem cells [1, 2]
- impaires blood cells in the course of immune mechanism [3], and
- changes the cell membrane [4, 5].

A relationship exists between exposure to organochlorine pesticides and their cumulation in adipose tissue, liver and bone marow on the one hand, and the development of the hemopoietic system disease on the other [6].

This paper describes the effect of lindane on blood cells, kidney and liver tissues after exposures of varying length of time.

Materials and Methods

Animals and animal exposure

Gamma-hexachlorocyclohexane (lindane) was synthetized by the Institute of Organic Industry in Warsaw (99.2% purity). Twenty-seven male rabbits chosen at random (mean body weight 2800 g) were administered lindane intra-

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gastrically every 24 hours. Lindane was dissolved in 1.5 ml of soyabean oil; the daily dose per rabbit was 7 mg/kg (0.1 LD_{50}) [7]. The control animals received the same volume of the solvent. Similar doses are used in the examination of chronic toxicological effects in experimental animals [8].

The experiments were performed in three different time series. The rabbits were administered:

1) a total of 0.5 LD_{50} lindane during one week, in equal doses,
2) a total of 0.5 LD_{50} lindane during one week, in equal doses; the rabbits were examined one week after the withdrawal of the pesticide,
3) a total of 2.0 LD_{50} lindane during 4 weeks, in equal doses (after one week −0.5 LD_{50}, after two weeks −1.0 LD_{50}, after three weeks 1.5 LD_{50} and after four weeks 2.0 LD_{50}).

Blood samples were taken once a week.

Cytotoxic effect

Erythrocyte, reticulocyte, leucocyte and differential white blood cell counts, haemoglobin level and haematocrite index were determined by routine methods in the rabbits' peripheral blood.

The physicochemical parameteres used for the characterization of erythrocytes were: osmotic fragility [9], and the degree of erythrocyte damage under conditions of "oxidative shock" measured by the spectrophotometric method. "Oxidative shock" was induced with 2.5 mg/ml of acetylphenylhydrazine (APH) at an incubation time of 3 hours. The levels of the methemoglobin and of denaturated haemoglobin were measured at the 630 nm wave and 700 nm wave, respectively.

The index of phagocytic activity and the percentage of non-phagocytizing neutrophils were calculated as described previously [10]. The nucleolar activity in the lymphocytes was determined according to Smetana [11].

The examined parameters of erythrocytes, neutrophils and lymphocytes are an important characteristic of these blood cell functions.

Prior to taking the liver and kidney sections, the animals were injected with pentobarbital, 100 mg per kg weight. The small fragments of liver and kidney were fixed in aceton at 4 °C, and embedded in paraffin. The tissues were cut into 7 μm thick sections and were stained by haematoxylin and eosin.

Statistical analysis

The statistical analysis was based on Student's *t* test, the calculation of ±SD and ±SEM and confidence extended to the difference between the average of two normal distributions at the confidence level of 5% [12]. The rise or fall of the mean values of the determined parameters was calculated as the per cent of mean control values (Δ%).

Results

In the course of the series of experiments, the weight of the experimental rabbits did not change as compared to the group of control animals. No changes were observed either in the haemoglobin level or in the haematocrit value.

After the administration of 2.0 LD_{50} lindane, the reticulocyte count rose by 25% in relation to the control group.

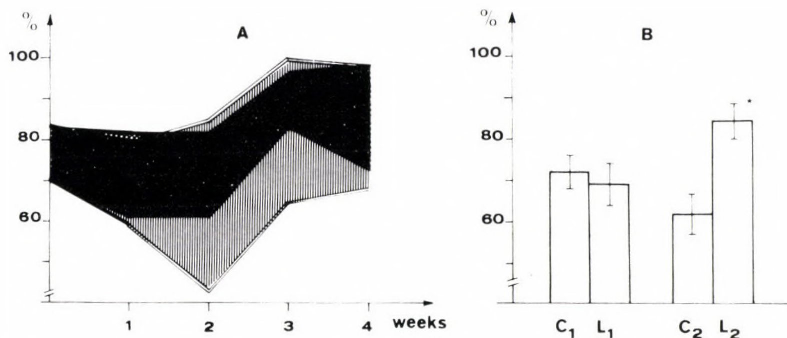


Fig. 1. Changes in the osmotic resistance (% hemolysis) of peripheral blood erythrocytes in rabbits (confidence extent at 5% level):
A — during four weeks of lindane administration, ▥ — Lindane, ▦ — Control;
B — after one week of lindane administration (1) and one week after lindane withdrawal (2); (C — control, L — lindane). Each bar represent mean ± SEM of five animals. Asterisks indicate statistically significant treatment effects

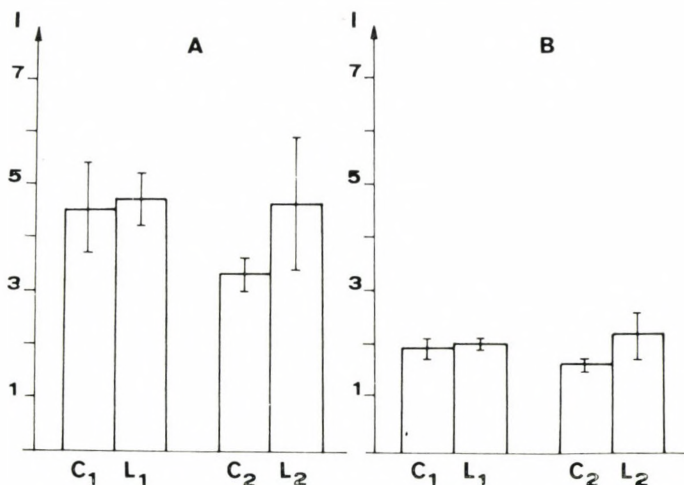


Fig. 2. Methemoglobin level (A) and denatured hemoglobin level (B) in peripheral blood rabbits' erythrocytes (C — control, L — lindane) after one week of lindane administration (1), and one week after lindane withdrawal (2), ($\bar{x} \pm$ SEM)
I— A and B values in relation to oxyhemoglobin accepted as 1

Some erythrocyte functions were changed, e.g. osmotic fragility (Fig. 1) and the sensitivity of the cells exposed to the "oxidative shock" (Fig. 2). After the administration of 1.0 LD_{50} lindane (two weeks), the osmotic fragility increased by about 10% over the control value, without changed sensitivity to the "oxidative shock". One week after the withdrawal of lindane, a significant decrease of osmotic fragility was observed, as evidenced by a 32% increase of haemolysis in relation to controls (Fig. 1b).

At the same time, the increased sensitivity of the erythrocytes to "oxidative shock" was expressed as a 42% rise (control I = 3.25, lindane I = 4.62) of the methaemoglobin level (Fig. 2a) and a 32% rise (control I = 1.63, lindane I = 2.16) of denaturated haemoglobin (Fig. 2b).

After the administration of 2.0 LD_{50} lindane, the leucocyte count decreased as compared to the control group.

The changes in leucocyte activity included a significant decrease in the phagocytosis index of the neutrophils (Fig. 3b), with an increase in the number of non-phagocytizing neutrophils after the administration of 0.5 LD_{50} lindane

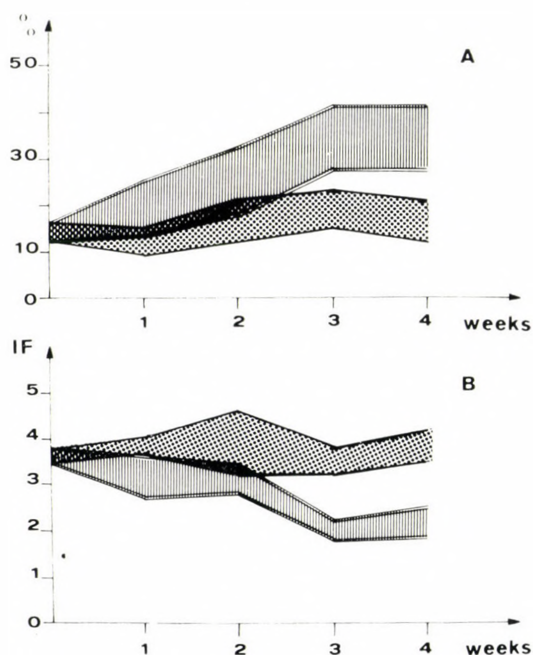


Fig. 3. Changes of the phagocytic activity of peripheral blood neutrophils in rabbits during four weeks of lindane administration
A — percentage of non-phygocytizing neutrophils,
B — phagocytosis index (IP) (confidence extent at 5% level)
▥ — Lindane, ▦ — Control

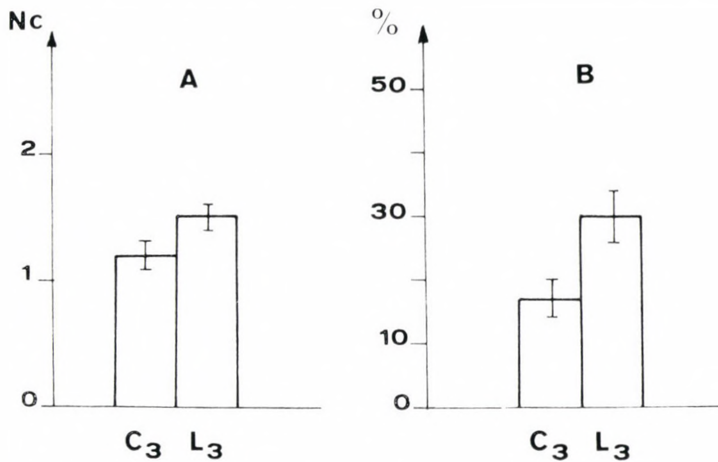


Fig. 4. Nucleoli in peripheral blood lymphocytes in rabbits after four weeks of lindane administration (3), (C — control, L — lindane). A — nucleolar coefficient (mean number of nucleoli in a lymphocyte $\bar{x} \pm$ SEM), B — percentage of lymphocytes with micronucleoli ($\bar{x} \pm$ SEM)

(Fig. 3a). The most significant decrease in phagocytosis was observed after the administration of 1.5 LD_{50} lindane (Fig. 3).

After the administration of 2.0 LD_{50} lindane, the lymphocyte population showed a significant rise in the percentage of lymphocytes with non-active nucleoli (micronucleoli) (Fig. 4b), which caused a significant increase in the lymphocyte nucleolar coefficient (Fig. 4a).

The administration of 2.0 LD_{50} lindane produced noticeable hepatocellular damage (Fig. 5), and frequent aggregation of erythrocytes in the hepatic lobules. In the renal cortex, the degeneration of glomeruli and tubular cells was observed (Fig. 6).

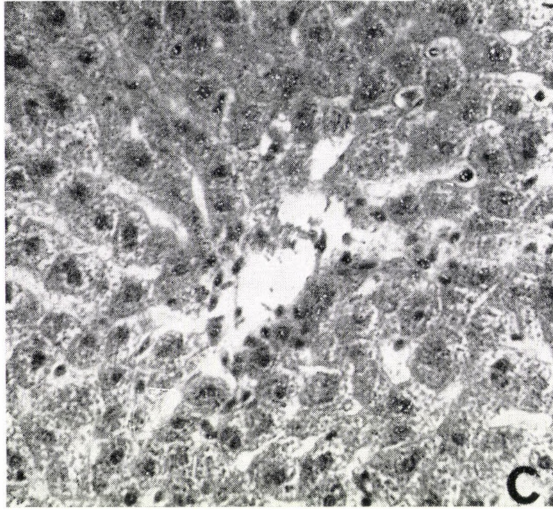
Discussion

The purpose of this paper was to explore the functional and structural changes in blood cells induced by lindane and to determine whether these alterations are toxic responses.

The focal lesions resulting in the degeneration of cell structure observed in liver and kidney, and the accompanying changes in the morphotic elements of the peripheral blood suggest that lindane has a cytotoxic effect.

The increase of the number of reticulocytes in the total erythrocyte pool observed after 4 weeks of lindane administration (2.0 LD_{50}) is a supplementation erythrocyte number reduction. This reduction is a result of increased phagocytosis of the erythrocytes by macrophages in the rabbits' liver [13].

In many cases, the borderlines between physiologic limits (normal functions) and toxicity (functional impairment) are difficult to define.



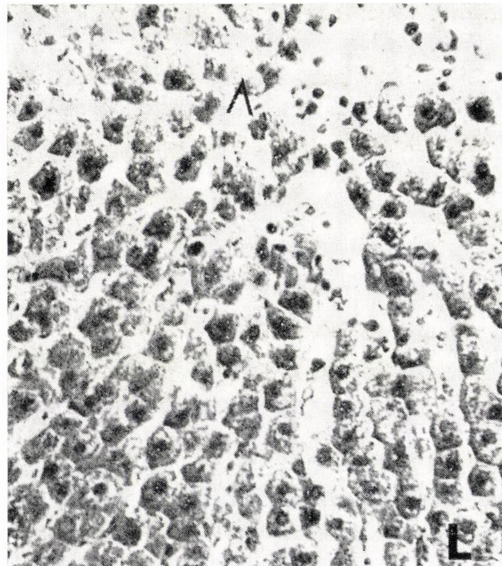
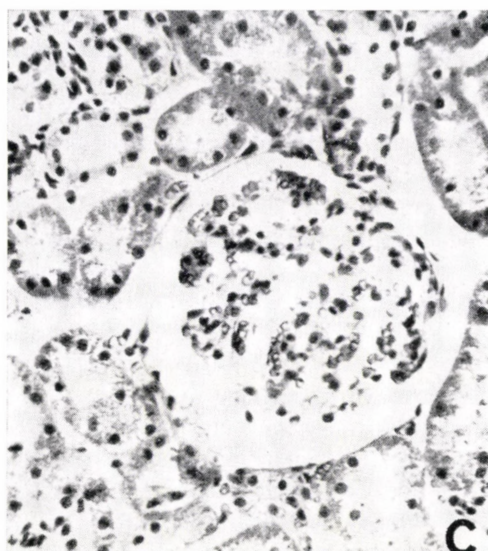


Fig. 5. The rabbit's liver after four weeks of lindane administration (2.0 LD_{50}); C — control, L — lindane: hepatocellular damage is visible (arrows), (Hematoxylin and eosin, 200×)



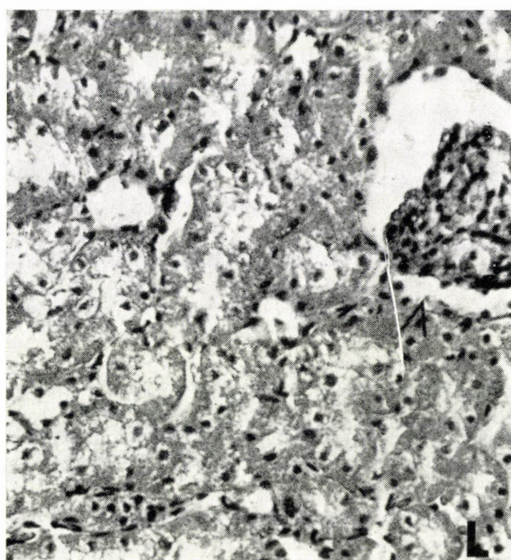


Fig. 6. Renal cortex of a rabbit after four weeks of lindane administration (2.0 LD_{50}); C — control, L — lindane: cells destruction in renal tubules is visible, glomerulus with changed structure (arrow), (Hematoxylin and eosin, 200×)

The impaired function of peripheral blood cells accompanying lindane administration was found to differ in red and in white blood cells. Changes in the physicochemical properties of the erythrocyte cell membrane varied during lindane administration and after its withdrawal, while the phagocytic activity of the neutrophils was decreased both during and after lindane administration. This observation can be connected to differences in the properties of the cell membrane structure of erythrocytes and neutrophils [14, 15]. It also depended on the similarity of lindane to inositol isomers, which are neutrophil cell membrane components [15].

The impairment of the neutrophils due to lindane is a cause of increased susceptibility to infections of the organisms exposed to this organochlorine pesticide [16].

The increased percentage of cells with non-active nucleoli observed after the administration of 2.0 LD_{50} lindane can be related to the inhibition of RNA-synthesis in the lymphocytes. A similar process was noted after the application of other pesticides [17] and cytostatic drugs [18].

The degenerative changes of renal tubules in the cortex observed after 2.0 LD_{50} lindane were similar to the effect obtained in the investigations of another chloriated compound [19], and are also connected with the process of the renal elimination of lindane metabolites [20].

The results obtained in the designed experimental set-ups suggest that further investigations of the cytotoxic effect of lindane on internal organs and peripheral blood are desirable; the changes in blood cells will be the "indicator" of the cytotoxic effects of lindane.

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Abstracts

Mobilization of iron from endocytic vesicles. The effects of acidification and reduction. M. T. Nuñez, V. Gaete, J. A. Watkins and J. Glass (Departamento de Biologia, Facultad de Ciencias, Universidad de Chile, Santiago, Chile). *J. Biol. Chem. 265*, 6688 (1990).

The factors necessary to dissociate iron from transferrin in endocytic vesicles and to mobilize the iron across the vesicle membrane were studied in a preparation of endocytic vesicles markedly enriched in transferrin-transferrin receptor complexes isolated from rabbit reticulocytes. Vesicles were prepared with essentially fully saturated transferrin by incubating the reticulocytes with the protonophore carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone prior to incubation with ^{59}Fe, ^{125}I-transferrin with or without fluorescein isothiocyanate labeling. Initiation of acidification by the addition of ATP was sufficient to achieve dissociation of ^{59}Fe from transferrin with a rate constant of 0.054 ± 0.06 s^{-1}. Mobilization of ^{59}Fe out of the vesicles required, besides ATP, the addition of a reductant with 1 mM ascorbate, allowing $\sim 60\%$ mobilization at 10 min with a rate constant of 0.0038 ± 0.0006 s^{-1}. An NADH: ferricyanide reductase activity could be demonstrated in the vesicles with an activity of 7.1×10^{-9} mol of NADH reduced per min/mg of vesicle protein. Both dissociation and mobilization were inhibited by N-ethylmaleimide, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, and monensin. Mobilization, but not dissociation, was inhibited by the permeant Fe(II) chelator α,α'-dipyridyl. The Fe(III) chelators deferoxamine, diethylenetriaminepentaacetic acid, and apotransferrin did not promote mobilization of dissociated iron in the absence of a reductant. This study establishes the basis for the cellular incorporation of iron through the endocytic pathway in which the endocytic vesicle membrane utilizes, in a sequential way, an acidification system, an iron reduction system, and an Fe(II) transporter system.

A. Egyed

Kinetic study of the Ca^{2+} pump in erythrocytes from essential hypertensive patients. A. De la Sierra, P. Hannaert, J. P. Ollivier, N. Senn and R. Garay (INSERM U7, Hôpital Necker, Paris 75015, France). *J. Hypertension 8*, 285 (1990).

Ca^{2+} pump kinetics were investigated in erythrocytes from 22 essential hypertensive patients and 20 normotensive controls (under initial-rate and steady-state conditions, using Sr^{2+} as a Ca^{2+} analogue). The mean value of the apparent dissociation constant for total internal Ca^{2+} (K_{Ca}) was slightly but significantly increased in the hypertensive population (73 ± 7 versus 55 ± 3 μmol/l cells, mean $\pm$ s.e.m., $P = 0.042$ Mann-Whitney U-test). The statistical analysis showed that this was due to six essential hypertensives who exhibited a dissociation constant for Ca^{2+} that was higher than the upper 95% normal confidence limit ($K_{Ca} = 116 \pm 7$ μmol/l cells), and abnormally high maximal pump rates (7.7 ± 0.6 versus 5.0 ± 0.2 mmol/l cells per h in normotensives, $P < 0.001$). In addition, the apparent dissociation constant for Ca^{2+} was inversely correlated with plasma renin activity, although the correlation was only borderline ($P = 0.076$). In the remaining 16 hypertensive patients, all kinetic parameters of the Ca^{2+} pump were within

the normal range. Finally, a simultaneous study of Na^+ transport kinetics suggested that erythrocyte Ca^{2+} and Na^+ transport abnormalities were independent phenomena. These results do not support the concept that primary hypertension (as a whole entity) is associated with a ubiquitous defect in the plasma membrane Ca^{2+} pump. However, in some essential hypertensive patients (about 25%) the erythrocyte Ca^{2+} pump exhibited an apparent decreased affinity for internal Ca^{2+}. A similar defect in vascular smooth muscle may induce a delayed Ca^{2+} extrusion after the opening of Ca^{2+} channels, a disturbance likely to be translated into increased vascular reactivity.

B. Sarkadi

Peroxidation-induced perturbations of erythrocyte lipid organization. D. Pradhan, M. Weiser, K. Lumley-Sapanski, D. Frazier, S. Kemper, P. Williamson and R. A. Schlegel (Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, PA, USA). *Biochim. Biophys. Acta 1023*, 398 (1990).

Peroxidation of erythrocyte membrane lipids by hydrogen peroxide perturbs the lipid bilayer and increases phagocytosis by macrophages. This study addresses the underlying mechanism of these processes, and in particular the role of malondialdehyde, a major byproduct of lipid peroxidation. When erythrocytes were treated with hydrogen peroxide or ascorbate/iron to generate malondialdehyde, on with malondialdehyde itself, only those cells treated with hydrogen peroxide showed increased phospholipid spacing and enhanced phagocytosis. This result indicates that the alterations observed are unique to hydrogen peroxide treatment, and that malondialdehyde does not play a role in inducing these changes in surface properties. Comparison of adherence to human umbilical vein endothelial cells and phagocytosis showed that increased phagocytosis was not mirrored by enhanced adherence. This result suggests that two different signals may mediate recognition of erythrocytes by macrophages and by endothelial cells.

G. Gárdos

The pH dependence of the hemolytic potency of bile salts. A. Ilani and R. Granoth (Department of Physiology, Hebrew University-Hadassah Medical School, Jerusalem, Israel). *Biochim. Biophys. Acta 1027*, 199 (1990).

The membrane damaging potential of dilute solutions of bile salts was evaluated by monitoring continuously the hemolysis of a small sample of red blood cells (RBC) introduced into a defined media containing the bile salts at various pH values. The strength of the hemolytic bile salt was characterized by the rate of the induced hemolysis and by the time that elapsed between the introduction of the RBC sample into the bile salt containing solution and the onset of hemolysis. The potency of the unconjugated bile acids was extremely sensitive to pH, e.g. the rate of hemolysis caused by a 7.5 mM cholate was 1.5%, 20% and 64% per min when the pH of the solution was 7.65, 7.3 and 6.85, respectively. At low pH values the membrane damaging effects of deoxycholate was clearly discerned at micromolar concentration range. The hemolytic potency of glycodeoxycholate was also enhanced significantly by lowering the pH. The taurine-conjugated cholate and deoxycholate were only slightly sensitive to variations in pH. Taurocholate at concentrations that were not hemolytic greatly enhanced the injurious potency of deoxycholate. These results imply that in acidic solutions the presence of bile acids can cause damage to cell membranes. It is suggested that the acidic environment in the proximal duodenum and acidosis developed during hypoxia in the liver are two situations in which the bile salts may constitute a pathogenic factor.

Ilma Szász

Mapping the binding sites of human erythrocyte ankyrin for the anion exchanger and spectrin. L. H. Davis and V. Bennett (Howard Hughes Medical Institute and Department of Biochemistry, Duke University Medical Center, Durham, North Carolina, USA). *J. Biol. Chem. 265*, 10589 (1990).

This report describes initial characterization of the binding sites of ankyrin for

spectrin and the anion exchanger using defined subfragnents isolated from purified ankyrin domains. The spectrin-binding domain of ankyrin is comprised of two subdomains: an acidic, proline-rich region (pI = 4) involving the amino-terminal 80 residues from 828 to 908 and a basic region (pI = 8.8) that extends from 898 to 1386. The amino-terminal 70 amino acids of the spectrin-binding domain are critical for association with spectrin, since a subfragment missing this region is only 5% as active as the intact domain in displacing binding of spectrin to inside-out membrane vesicles, while deletion of the first 38 residues of the acidic domain results in a 10-fold reduction in activity. The anion exchanger-binding site is confined to an 89-kDa domain that was isolated and characterized as a globular molecule with approximately 30% α-helical configuration. A subfragment of the 89-kDa domain extending from residues 403 to 779 (or possibly 740) retains ability to associate with the anion exchanger. The 89-kDa domain is comprised of a series of tandem repeats of 33 amino acids that extend from residues 35 to 778. The activity of residues 403—779 demonstrates that the 33-amino acid repeats of the 89-kDa domain are responsible for association between ankyrin and the anion exchanger. The 33-amino acid repeating sequence of ankyrin represents an ancient motif also found in proteins of *Drosophila*, yeast, and *Caenor habditis elegans*. The finding that the 33-amino acid repeating sequence is involved in interaction with the anion exchanger implies that this motif may perform a role in molecular recognition in diverse proteins.

B. Sarkadi

ESR analysis with long-chain alkyl spin labels in bovine blood platelets. Relationship between the increase in membrane fluidity by alcohols and phenolic compounds and their inhibitory effects on aggregation. Sh. Kitagawa, F. Kametani, K. Tsuchiya and H. Sakurai (Laboratory of Physical Chemistry, Faculty of Pharmaceutical Sciences, University of Tokushima, Tokushima, Japan). *Biochim. Biophys. Acta 1027*, 123 (1990).

Four spin-labeled probes (5-doxylstearic acid (5-NS), its methyl ester (5-NMS), 16-doxylmethylstearate (16-NMS) and 4-(N,N-dimethyl-N-pentadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl (CAT-15) were used to monitor membrane fluidity change in bovine platelets induced by three alkyl alcohols, benzyl alcohol and two phenolic compounds. The relationship between the inscrease in membrane fluidity induced by these compounds and their inhibitory effects on platelet aggregation was observed. Experiments with the four probes showed that n-hexyl alcohol induced decreases in the order parameter of 5-NS and apparent rotational correlation times of the other probes at the same minimal alcohol concentration. The decreases were observed in the concentration range that inhibited aggregation. n-Amyl alcohol and n-butyl alcohol decreased the values of the parameters of the above mentioned only at higher concentrations that were dependent on their hydrophobicities. Like alkyl alcohols, benzyl alcohol and phenolic compounds decreased the values of the parameters in the concentration ranges in which these compounds inhibited platelet aggregation. The concentration of these compounds causing 50% inhibition of platelet aggregation, the IC_{50} values, and data on 5-NS-labeled platelets indicated that they inhibited aggregation and decreased the value of the order parameter at lower concentrations relative to their P_{oct} values in comparison to the effective concentrations of alcohols. Phenolic compounds also decreased the values of the apparent rotational correlation times of 5-NMS and 16-NMS. These results indicate that the inhibition of platelet aggregation by alcohols and phenolic compounds is due to membrane perturbation in wide range in depths within the lipid bilayer.

Ilma Szász

Divalent cation-dependent structure in the platelet membrane glycoprotein Ia—IIa (VLA-2) complex. W. D. Staatz, K. J. Peters and S. A. Santoro (Division of Laboratory Medicine, Departments of Pathology and Medicine, Washington University School of Medicine, St. Louis, MO, USA). *Biochem. Biophys. Res. Comm. 168*, 107 (1990).

Recent studies have shown that the platelet membrane glycoprotein Ia—IIa (VLA-2) complex mediates the Mg^{++}-dependent adhesion of platelets to collagen and that this adhesion is inhibited by Ca^{++} in a simple, linear, noncompetitive manner. These findings suggested that separate binding sites for Mg^{++} and Ca^{++} stabilize different divalent cation-dependent structures within the receptor complex. To provide evidence for the existence of such structures purified platelet Ia—IIa complex was subjected to limited proteolytic digestion in the presence of Mg^{++}, Ca^{++}, Mg^{++} and Ca^{++}, or EDTA and the resulting peptides mapped by SDS-PAGE using both one- and two-dimensional techniques. Unique patterns of tryptic peptides were produced under each of the conditions. The results indicate that Mg^{++} and Ca^{++} stabilize different structures within the Ia—IIa (VLA-2) complex and that these structures influence both the collagen binding activity and proteolytic susceptibility of the complex.

G. Gárdos

Decorsin. A potent glycoprotein IIb—IIIa antagonist and platelet aggregation inhibitor from the leech Macrobdella decora. J. L. Seymour, W. J. Henzel, B. Nevins, J. T. Stults and R. A. Lazarus (Departments of Biomolecular Chemistry and Protein Chemistry, Genentech, Inc., South San Francisco, California, USA). *J. Biol. Chem. 265*, 10143 (1990).

The discovery, purification, and characterization of decorsin, a protein isolated from the North American leech Macrobdella decora, are described. Decorsin acts as an antagonist of platelet glycoprotein IIb—IIIa (GPIIb—IIIa), and is a potent inhibitor of platelet aggregation. The protein was purified to apparent homogeneity from crude whole leech extracts by treatment with trifluoroacetic acid followed by GPIIb—IIIa affinity chromatography and C_{18} reverse-phase high performance liquid chromatography. Decorsin was also isolated from a solution of leech ingestate by treatment with trifluoroacetic acid followed by C_{18} reverse-phase high performance liquid chromatography. The primary sequence of decorsin indicates that the protein is 39 amino acids long and contains 6 cysteine and 6 proline residues, as well as the sequence Arg-Gly-Asp, (RGD), a proposed regognition site of many adhesion proteins. A molecular mass of 4379 was obtained by fast atom bombardment mass spectrometry and is consistent with the mass calculated from the observed sequence. Evidence for an N-3 isoform, lacking the first 3 amino-terminal residues is also presented. Both decorsin and the N-3 isoform inhibit GP IIb—IIIa binding to immobilized fibrinogen with an IC_{50} of $\sim$ 1.5 nM. Human platelet aggregation induced by ADP is inhibited by decorsin with an IC_{50} of $\sim$ 500 nM; complete inhibition was observed at $\leq 1\ \mu$M. Based on overall sequence homology, decorsin does not belong to the family of GPIIb—IIIa protein antagonists that is found in snake venoms, however, the carboxyl-terminal RGD-containing region from residues 27 to 38 of decorsin is approximately 60% homologous with the corresponding region of the snake venom proteins, suggesting that high affinity binding of these proteins to GPIIb—IIIa is defined by this epitope.

G. Gárdos

Membrane depolarization selectively inhibits receptor-operated calcium channels in human T (Jurkat) lymphoblasts. B. Sarkadi, A. Tordai and G. Gárdos (National Institute of Haematology and Blood Transfusion, Budapest, Hungary). *Biochim. Biophys. Acta 1027*, 130 (1990).

Jurkat lymphoblasts were stimulated by a monoclonal antibody against the CD3 membrane antigen and the evoked calcium signal was followed by the intracellular fluorescent calcium indicator indo-1. The technique applied allowed us to separately investigate the stimulus-induced intracellular calcium release and the calcium-influx pathways, respectively. In the same cells membrane potential was estimated by the fluorescent dye diS-C_3-(5). The resting membrane potential of surkat lymphoblasts under normal conditionJ was between -55 and -60 mV. Membrane depolarization, obtained by increasing external K^+ con-

centration, removing external Cl^-, or by increasing the Na^+/K^+ leak permeability with gramicidin or PCMBS, did not induce calcium influx in the resting cells and did not influence the CD3 receptor-mediated internal calcium release, while strongly inhibited the receptor-mediated calcium influx pathway. Half-maximum inhibition of this calcium influx was observed at membrane potential values of about −35 to −40 mV and this inhibition did not depend on the external calcium concentration varied between 5 and 2500 μM. Membrane hyperpolarization by valinomycin did not affect either component of the calcium signal. The observed selective inhibition of the receptor-operated calcium influx pathway by membrane depolarization is probably an important modulator of calcium-dependent cell stimulation.

Ilma Szász

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Ethanol Decreases the Level of Sulfhydryl Compounds in Red Blood Cells and Changes Their Hematological Pattern in Rats

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The level of sulfhydryl compounds in red blood cells (RBCs) and plasma and some haematological parameters were investigated in rats treated with ethanol for 4 weeks (daily dose of 6g/kg, 30% w/v, p.o.). After ethanol ingestion, the significant decrease of non-protein –SH groups were observed in RBCs and plasma. In treated rats, the mean corpuscular haemoglobin concentration (MCHC) was decreased and the mean corpuscular volume (MCV) was increased. There is a positive correlation between these two parameters and decreased content of sulfohydryl groups in RBCs.

Keywords: ethanol, haematological pattern of blood, plasma, rats, red blood cells, sulfhydryl compounds

Introduction

Sulfhydryl groups are constant constituents of all cells and necessary for their integrity and normal function. In erythrocytes, they are involved in reactions maintaining the native structure of haemoglobin, enzimes and membrane proteins [1]. Furthermore, SH groups participate in the protection of cells against free radicals and toxic compounds of endogenous and exogenous origin [2].

Acute and chronic ethanol (Et-OH) administration was found to induce alteration in hepatic reduced glutathione (GS-H) [3–4] hepatic non-protein sulfhydryl compounds and plasma protein sulfhydryl compounds [5]. The significant decrease in the content of protein and non-protein SH groups in liver and plasma was observed after acetaldehyde ingestion [6].

The aim of the present study was to investigate the effect of Et-OH on the level of sulfhydryl compounds of the red blood cell nonprotein fraction and on haematological pattern of rat blood.

Materials and Methods

Male Wistar rats (approx. 200 g) fed the standard diet, were divided into two groups: (A) 15 rats were given a daily dose of 6 g/kg ethanol (30% w/v in isotonic NaCl) p.o. for 4 weeks, (B) 10 rats (control group) received an equivalent volume of 0.15 M NaCl.

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Blood for haematological measurements was taken by cardiac puncture into heparinized containers. The erythrocytes were separated from the plasma by centrifugation. The level of SH groups was measured in plasma and in haemolysed and deproteinized erythrocytes using 5,5'-dithiobisnitrobenzoic acid [7].

The following investigations were carried out: total red cell count (RBCs) was determined by chamber method, haemoglobin level (Hb) – by the cyanmethaemoglobin method and haematocrit (HCT) – by the micromethod. The mean corpuscular volume (MCV) and corpuscular haemoglobin concentration (MCHC) were calculated.

In serum, the activities of aspartate aminitransaminase (AspAT) [8] and γ-glutamyltranspeptidase (GGTP) [9] were estimated.

The obtained results were analysed statistically with Student's *t*-test. The value of $p < 0.05$ was taken as a statistically significant difference.

Results

Sulfhydryl compound level in erythrocytes and plasma of rats given ethanol for 4 weeks and of control group are summarized in Table 1. The amount of SH groups was about 35.4% lower in the erythrocyte non-protein fraction of rats treated with ethanol than in the control group. The differences were statistically

Table 1

The effect of ethanol on non-protein sulfhydryl groups in RBCs and in the blood plasma of rats

	Sulfhydryl compounds in	
	RBCs μM/ml	plasma μM/ml
Ethanol group	0.410 + 0.094*	0.187 + 0.077*
Control group	0.634 + 0.081	0.271 + 0.095

* $p < 0.05$ with respect to the control group

significant ($p < 0.05$). The total level of sulfhydryl compounds in the plasma of rats treated with ethanol was also significantly decreased (31%) ($p < 0.05$).

Table 2 summarizes the haematological parameters estimated in the rats treated with ethanol and in the control group. In rats treated with ethanol, the increase of MCV and decrease of MCHC were observed. The differences were statistically significant. The ethanol group showed the enhancement of haematocrit value. Nevertheless, the erythrocyte count and the haemoglobin level did not differ from the control values.

Table 2

The effect of ethanol on the haematological parameters

	Control group	Ethanol group
Erythrocyte count (mln/μl)	11.06 + 0.87	10.64 + 0.77
Hematocrit (%)	44.70 + 1.80	48.90 + 2.30*
Hemoglobin (g/l)	146.60 + 4.30	125.30 + 22.50
MCV (μm^3)	40.41 + 3.21	45.95 + 2.17*
MCHC	32.79 + 0.63	25.66 + 1.35*

* $p < 0.05$ with respect to the control group

Table 3

The effect of ethanol on rat serum aspartate aminotransferase and γ-glutamyltranspeptidase

	Control group	Ethanol group
AspAT (IU)	155.0 + 21.5	195.9 + 11.9*
GGTP (IU)	30.5 + 10.1	46.9 + 7.5*

* $p < 0.05$ with respect to the control group

In rats treated with ethanol, the activities of AspAT and of GGTP were significantly increased (Table 3).

Discussion

Erythrocytes subjected to various physical and chemical factors can be rapidly transformed into a broad spectrum of transitional shapes that may alter the haemodynamic properties of blood and decrease cell survival in the circulation [10]. The lipid bilayer of the erythrocyte membrane [11], and/or its underlying cytoskeletal protein network may be directly responsible for the control of cell shape and specific rheologic properties. As one of the world's most widely used drugs, ethanol is known to have deleterious effects on many organs and also on the blood cells [11]. Experiments have shown that Et-OH exerts its pharmacological effects mainly by changing the "fluidity" [13]. Recent studies have docu-

mented that Et-OH induces alteration in the membrane lipid composition of red blood cells (RBCs), due to decreased cholesterol efflux [12]. Ethanol induced alterations in the shape of human RBCs were accompanied by an enhancement of agglutination mediated by ligands with high affinity for negative cell surface charge [14].

In our studies, the significant decreases in sulfhydryl compounds of erythrocyte non-protein fraction was observed in rats treated with Et-OH for 4 weeks. The non-protein sulfhydryl groups consist mainly of the naturally occurring tripeptide: glutathione (GSH), free amino acid cystein and other components containing free SH groups. In RBCs, the level of tripeptide is about 1000 times greater than in plasma, however, in erythrocytes SH groups are present in their reduced form [15].

The diminution of erythrocyte SH groups results in oxidative damage of cell [16] and defective transport of some amino acid into the cell. Expression of amino acid transport deficiency was shown to precede the decrease in intracellular SH groups. These groups participate in the degradation of hydrogen peroxide, which is continuously formed in little amounts in the course of metabolic pathway and also under the influence of some drugs. They also protect haemoglobin against oxidative denaturation of its protein.

Our results are in agreement with those obtained by Uysal et al., who have studied erythrocyte glutathione level in chronic alcoholic patients [17].

The decrease in the level of sulfhydryl compounds content in non-protein fraction of RBCs was accompained by changes in other haematological parameters. Our studies show positive correlation between the decrease in SH groups and diminution of MCHC in rats treated with Et-OH.

It can be concluded that observed abnormalities in intracellular non-protein sulfhydryl compounds of RBCs are responsible for the morphological characteristics of subsequently altered physico-chemical properties of these cells. It is possible that these observations may also be useful as markers of alcohol abuse.

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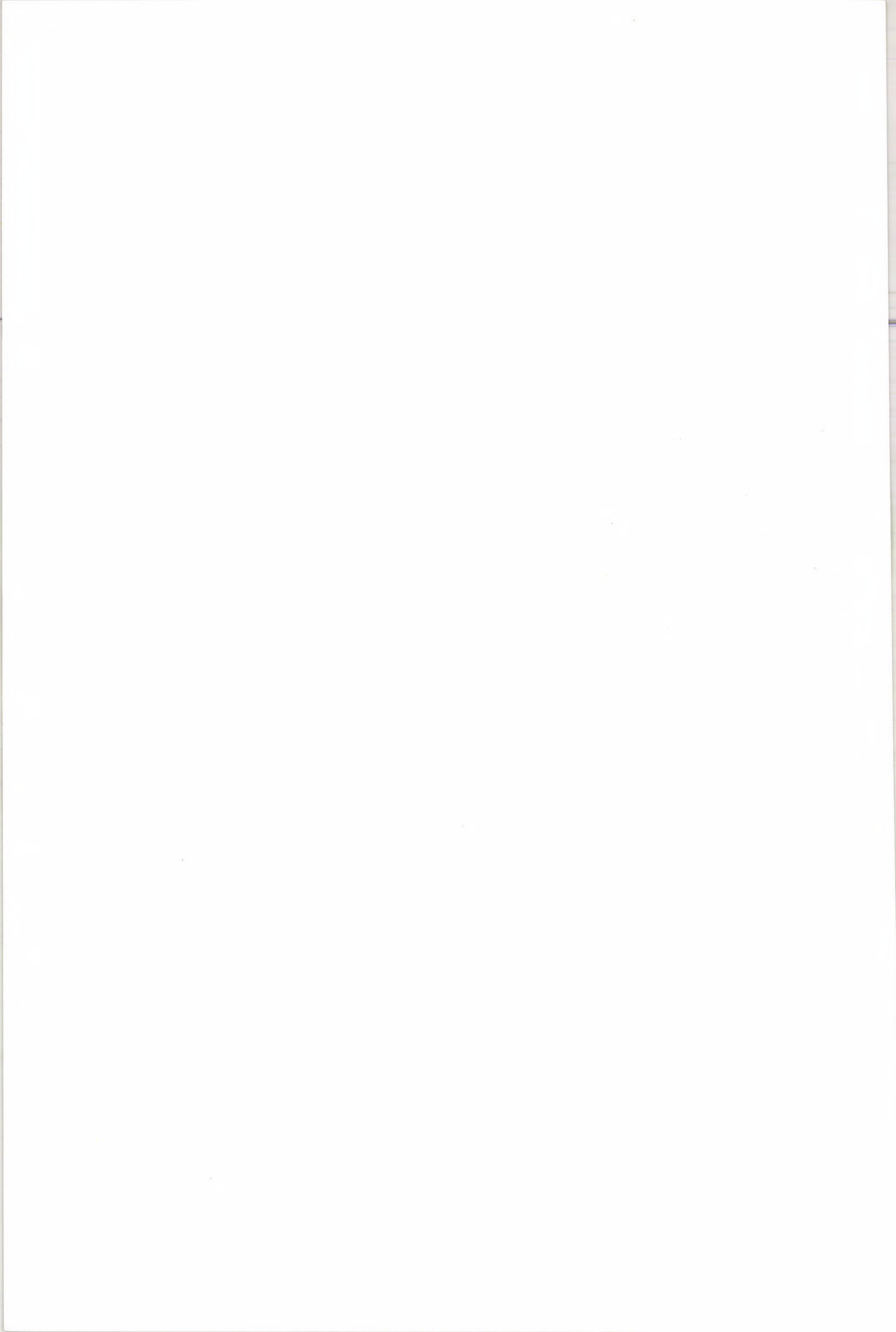

Haematologia 23 (4), pp. 193 – 197 (1990)

Activation Antigens in Patients with Sjögren's Syndrome

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(Received 16 November, 1989; accepted 22 January, 1990)

We report findings of a study of two receptors on mononuclear cells from patients with Sjögren's syndrome (SS). The two receptors, interleukin-2 receptor (IL-2), transferrin receptor (TfR), were identified using monoclonal antibodies anti-IL-2 and OKT9.

We found that the IL-2 and TfR positive cells were significantly higher on mononuclear cells (MC) from peripherial blood (PB) of patients with SS than in healthy controls.

The study revealed that the IL-2 and TfR positivity reflects systemic immune activation and correlates closely with the activity of SS.

Keywords: Sjögren's syndrome, clinical activity, interleukin-2 receptor, transferrin receptor

Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disease characterised by lymphoid cell infiltration of exocrine glands, especially lacrimal and salivary glands, resulting in xerostomia and xerophthalmia [14, 22, 24].

Patients with SS have features suggestive of B cell hyperreactivity: including polyclonal hypergammaglobulinaemia, the presence of various autoantibodies (such as rheumatoid factor and anti-nuclear antibodies) and circulating immune complexes [1, 2, 11].

Data of previous studies suggest that the T lymphocytes are activated in patients with SS [12, 25]. Resting T cells do not express high-affinity IL-2 receptors; however, after activation with an antigen or mitogen, T cells rapidly express receptors. The IL-2 receptor (IL-2R) is composed of at least two subunits, the p55 (CD25-Tac) and the p75 glycoproteins. p55 and p75 both bind IL-2 independently with either low or intermediate affinity, whereas a heterodimeric receptor composed of p55 and p75 binds IL-2 with high affinity.

Our standard approach for the identification of T cell activation depends on detecting CD25-Tac and the transferrin receptor (TfR-CD71).

We wanted to find a fast laboratory test to determine clinical activity of SS. We measured the IL-2R and TfR positivity on the PBMC and determined whether they correlated with disease activity.

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Materials and Methods

Patients

Twenty-one patients with SS were studied. 21 females, aged 39 to 68 years (average 52 years). SS was diagnosed according to previously established criteria [5].

Keratoconjunctivitis sicca was diagnosed after complete ophthalmologic examination. Xerostomia was established on the basis of a reduction in the stimulated or spontaneous parotid flow rate and a focus score of more than 1 on labial salivary gland biopsy [6]. Nineteen SS patients had only glandular (primary SS) and another two had SS with an associated connective tissue disease (rheumatoid arthritis: RA). RA was diagnosed by standard diagnostic criteria [20]. SS of ten patients were in active clinical stage [26].

The control group was age and sex matched.

Isolation of lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by density centrifugation on Ficoll-Hypaque as previously described by Miyasaka et al. [15]. The mononuclear cells were collected from the interface, washed three times, and resuspended in complete RPMI-1640 (GIBCO) containing 100 μg/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamin, 10 mM Hepes supplemented with 1% fetal calf serum.

Monoclonal antibodies, indirect immunofluorescence, FACS analysis

Anti-Tac (Becton Dickinson) and anti-transferrin (OKT9, Ortho Diagnostic System) monoclonal antibodies were used to detect IL-2 (CD25) and TfR (CD71) human lymphocyte surface antigens. 100 μl PBS containing 5% rabbit serum and 5 μl monoclonal antibodies were added to 100 μl 1×10^7/ml of PBMC suspension. It was incubated in an ice water bath, 4 °C, for 30 min, then washed in PBS three times. Cells were labelled by 100 μl FITC-conjugated-anti-mouse-IgG. The cells were rewashed three times and fixed by 0.04% formaldehyde. The marked cells were analysed on a FACS-star. After 10,000 cells had been counted, the number of fluorescent cells was given in percentage [9]. Anti-mouse immunglobulin (DAKO) produced in rabbit and marked with FITC was applied as a second antibody for the indirect test.

Results

The mean ($\pm$ SE) positivity of IL-2R on PBMC from the 11 normal control subjects was 3.1 $\pm$ 1.4. Table 1 shows that the IL-2R positivity were markedly elevated in patients with SS if the disease was in a clinically active stage (18.48 $\pm$

Table 1

Interleukin-2 receptor (IL-2R) and transferrin receptor (TfR) positivity on peripheral blood mononuclear cells from patients with Sjögren's syndrome

Examined markers	I Sjögren's syndrome in active clinical stage (n = 10) % of positive cells (mean ± SE)	II Sjögren's syndrome in inactive clinical stage (n = 11) % of positive cells (mean ± SE)	III Control (mean ± SE)	P value
IL-2R	18.48±5.1	2.72±0.5	3.1±1.4	$P_{I-II} = 0.01$ $P_{I-III} = 0.01$ P_{II-III} = N.S.
TfR	25.81±9.68	2.61±0.55	3.7±0.7	$P_{I-II} = 0.02$ $P_{I-III} = 0.02$ P_{II-III} = N.S.

± 5.1), p value v.s. control was < 0.01 (by Student's *t*-test). The mean (± SE) positivity of IL-2R was 2.72 ± 0.55 if the SS was in an inactive clinical stage, and did not differ significantly from the positivity of the normal controls (Table 1). IL-2R positivity correlated with the clinical disease activity in patients with SS.

Markedly elevated TfR positivity was found on PBMC of patients with SS (patients in active clinical stage: 25.81 ± 9.68; controls: 3.7 ± 0.7). TfR positivity reflected the clinical activity.

Discussion

SS is considered to be associated with immune system activation. Likewise, SS is considered to be a disease caused by unregulated immune system activation resulting from as yet unidentified antigen(s).

Activated T cells [19], B cells [23, 27], natural killer cells [13], and macrophages [7, 8] express IL-2 receptors.

It is known that maximal expression of cell membrane IL-2 occurs after 48 – 72 hours [4] and that levels of IL-2R peak after 72 hours of in vitro activation with mitogens [17, 18].

Previously reported studies of the TfR have suggested that the appearance of this receptor is a prerequisite for cells to proceed from G1 phase to S phase in a cells DNA cycle [10]. Neckers and Cossman reported that the IL-2R needed to be expressed on phytohaemagglutinin in assay stimulated lymphocytes before the TfR could be expressed [16]. Work by Bettens et al. on lymphocytes stimulated by phytohaemagglutinin in assay showed that IL-2R is required to convert G_{1a} (medium RNA content) into G_{1b} (high RNA content) [3]. There could, therefore, be an evolutionary link between the two receptors.

We report the findings of a study of two receptors on PBMC from patients with SS. IL-2R and TfR positive cells were found to be significantly increased in PBMC of patients with SS. The study revealed that the IL-2R and TfR positivity reflects systemic immune system activation and correlates closely with the activity of SS.

The ESR (erythrocyte sedimentation rate) is generally considered to be the best available serologic marker for monitoring disease activity in patients with autoimmune disease [21]. In some patients whose SS is in full clinical remission, a persistently elevated ESR may be present, even though all other laboratory results are normal.

Our findings indicate that serum levels of IL-2R and TfR may serve as a reliable serologic indicator of disease activity in inflammatory diseases characterized by immune system activation.

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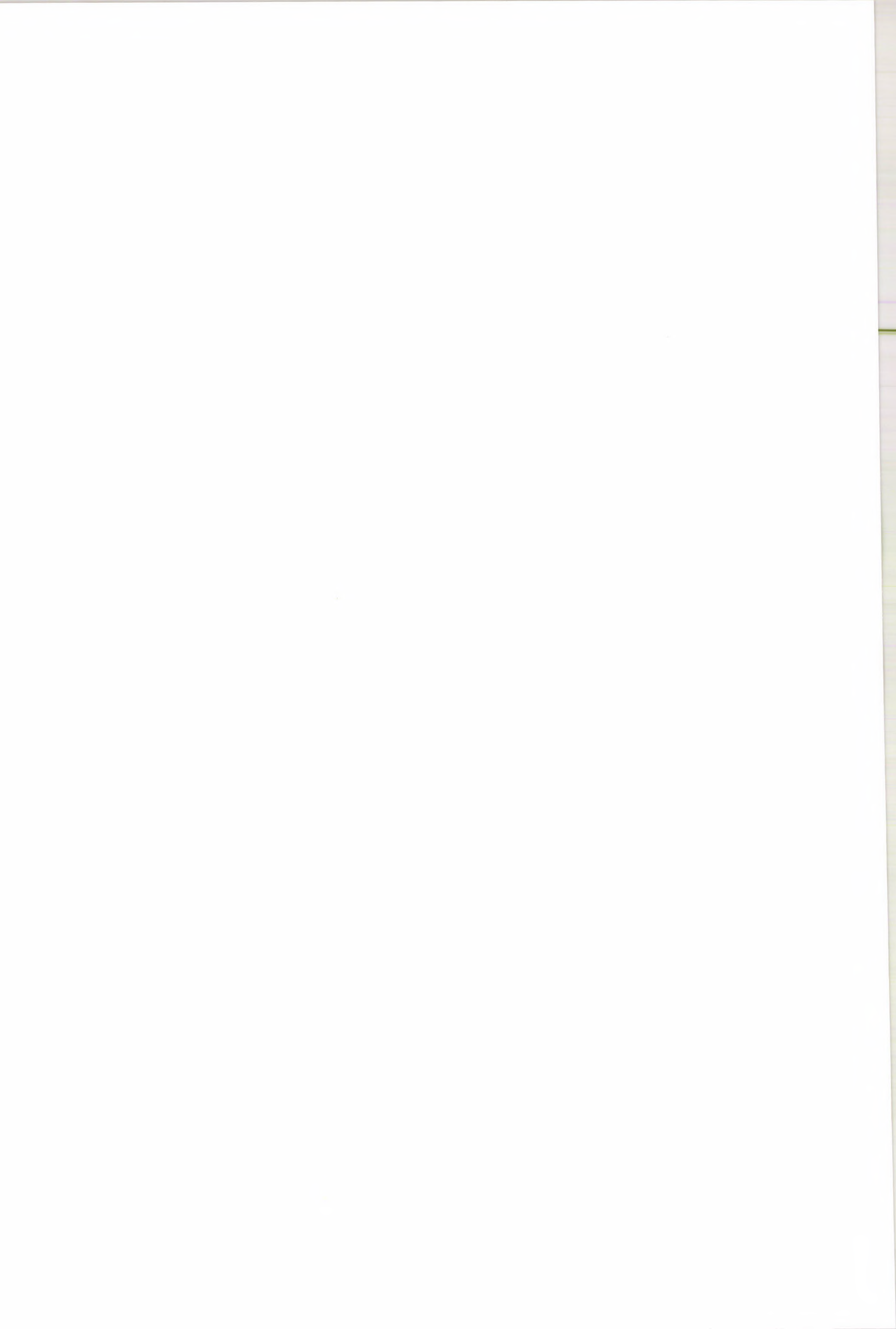

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Clinical Value of Cytomorphologic, Immunologic and Cytogenetic Investigations of Acute Leukaemias

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The valuability of immunophenotyping of acute myeloid and lymphoid leukaemias in comparison to morphological and cytochemical classification were approached in 56 cases. In the case of acute myeloid leukaemias the immunophenotyping by monoclonal antibodies CD14, CD13, CD33 was less informative concerning the subtypes of the disease. The clinical diagnosis can be achieved on the basis of cytochemical investigation alone. In contrast, the diagnosis of lymphoid leukaemias requires all information obtained by immunophenotyping by a series of monoclonal antibodies CD3, CD2, CD4, CD8, CD1, CD19, CD20, CD21 and CD10. On the other hand, the monoclonal antibodies are essential in differentiation of the very immature myeloid and lymphoid leukaemias. This is of great importance from the clinical point of view for determining the therapy. Molecular genetic studies based on the characterisation of the state of gene rearrangement of immunoglobulin and T-cell receptor beta chains have basic importance in the confirmation of the result of immunophenotyping and in the determination of leukaemias of unknown origin.

Keywords: FAB classification, immunophenotyping, gene rearrangement

Introduction

Conventional classification of the acute leukaemias as defined by the French–American–British (FAB) cooperative group is based on the morphological and cytochemical characteristics of the blasts [1]. Using these criteria approx. 70–90% of the acute leukaemias (ALs) are of myeloid-monocytic or lymphoid origin [2]. Over the last decade, however, major advances in the characterisation of leukaemic blasts have been made with the use of monoclonal antibodies specific to various stages of differentiation of the lymphoid and myeloid lineages [3, 4, 5, 6]. Immunophenotypic analysis can be used successfully: 1) to classify most cases which cannot be differentiated by cytochemistry [7], 2) to subclassify the acute lymphoid leukaemias (ALLs) into prognostically important subgroups and 3) to identify myeloid, monocytic and other non-lymphoid blast cells [8, 9, 10, 11, 12, 13, 14, 15]. However, the discrimination between B-cell and T-cell lineage may be inconclusive when the neoplastic cells are a mixture of blasts from different lineages or/and from biphenotypic leukaemias [16, 17]. For this reason, the analysis of immunoglobulin gene and T-cell receptor gene rearrangement be-

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came increasingly important in the study of leukaemic cell clonality and lineage [18, 19, 20].

In this work we examined the immunological phenotype of peripheral blood mononuclear cells in a group of acute lymphoid leukaemia (ALL) and acute myeloid leukaemia (AML) patients in respect of clinical benefit. Results were compared with data obtained by FAB classification and cytochemical stains and in a few cases with the rearrangement of immunoglobulin and T-cell receptor genes.

Materials and Methods

Patients: Fifty-six patients (33 males and 23 females) affected by AML or ALL were studied. Mean age was 35 yrs (range 14–73). All patients were studied before initiation of induction chemotherapy.

Diagnosis and classification: The preliminary diagnosis and classification were performed using the FAB criteria. The morphology of the leukaemic blasts was analysed in May–Grünwald–Giemsa stained smears. The following reactions were performed: periodic acid Schiff (PAS), acid phosphatase (AP), Sudan black B (Sudan BB), naphthol-ASd-choloacetate esterase (NASDCA), deoxyribonuclease (DN-ase), alpha-naphtyl-acetate-esterase (ANAE), myeloperoxidase (MPO) and muramidase. Smears obtained by bone marrow aspiration were used for each reaction.

Immunological analysis: Peripheral blood and in few cases also bone marrow were used for immunological analysis. Mononuclear cells obtained after Ficoll–Hypaque gradient centrifugation were tested with a commercially available monoclonal antibody panel by indirect immunofluorescence in flow cytometry. T-cell associated antibodies were CD3(T3), CD2(T11), CD4(T4), CD8(T8), CD1(T6), from Ortho Diagn. System. The B-cell associated reagents were CD19(B4), CD20(B1), CD21(B2), CD10(J5) from Coulter Corporation, CD22(RFB4) from the Royal Free Hospital. Myeloid associated reagents were CD13(My7), CD33(My9), CD14(MO2) from Coulter Corp. Class II. antigens were detected with HLA-DR from Becton–Dickinson. Anti-IgM antibody (Heintel) was used as directly conjugated with FITC. In the indirect immunofluorescence FITC conjugated rabbit anti-mouse immunoglobulin was used as second antibody (DAKO). The cell surface marker determination was performed according to the method of Janossy et al. [21]. Analysis of all samples was done using FACSTAR flow cytometer (Becton–Dickinson). The different right and forward angle light-scattering of normal and blast cell was also used to separate the two cell fractions. The relative fluorescence intensity of 10 000 cells per sample was determined. Intracytoplasmic CD3 and CD22 stains were used according to the method of Janossy et al. [11, 12, 21]. The diagnosis was confirmed by analysing the immunoglobulin (Ig) and T-cell receptor beta chain gene (TCR) rearrangement [22, 23]. The immunologic subclassification of leukaemias was performed according to the criteria of Foon and Todd [8].

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Table 1

Cytochemical and immunological results of patients with AML I

Patients	MPO	SUDAN BB	DN-ase	NASDCA	ANAE	FAB	CD14 MO2 %	CD13 MY7 %	CD33 MY9 %	HLA-DR %	Immunol. Phenotype
1.	+++	+++	+−	+++	++	M4	11	12	21	nt.	myeloid
2.	++	+++	+	+++	++	M5	6	16	44	nt.	myeloid
3.	−	−	−	−	−	M0	4	32	0	61	myeloid
4.	+++	+++	−	+++	+++	M4	5	56	40	22	myeloid
5.	+++	++	+++	+++	+++	M4	17	12	8	50	myeloid
6.	+++	+++	+++	++	++	M4	1	14	80	54	myeloid
7.	−	−	−	−	+	M0	1	4	100	1	myeloid
8.	−	−	−	−	+	M0	3	9	10	68	stem cell
9.	+++	+++	+++, +	+++, +	++	M4	23	87	15	71	myeloid
10.	+++	++	+++, +	++	+	M2	8	75	nt.	73	myeloid
11.	+	+	+, +++	+	+	M4	5	34	98	5	myeloid
12.	+++	+++	+++	+++	+	M2	30	34	nt.	69	myeloid
13.	++	++	++	++	+	M2	42	37	nt.	20	myeloid
14.	++	++	+−	+	+−	M1	22	86	46	17	myeloid
15.	+++	++	+−	++	+	M4	8	87	nt.	83	myeloid
16.	+++	+++	+++	+++	+−	M2	8	25	nt.	63	myeloid

nt. = not tested

Table 2

Cytochemical and immunological results of patients with AML II

Patients	MPO	SUDAN BB	DN-ase	NASDCA	ANAE	FAB	CO14 MO2 %	CD13 MY7 %	CD33 MY9 %	HLA-DR %	Immunol. Phenotype
17.	+++	+++	+	+++	+	M4	nt.	nt.	22	72	myeloid
18.	–	–	+	–	+–	M0	4	14	11	41	stem cell
19.	–	–	–	+	–	M0	8	9	nt.	50	stem cell
20.	+	–	–	+	–	M0	1	62	nt.	62	myeloid
21.	+	++	+	++	++	M4	33	45	nt.	24	myeloid
22.	++	++	+–	++	+	M1	32	28	62	60	myeloid
23.	++	+	+–	+	+	M2	4	nt.	70	50	myeloid
24.	+++	+++	++	++	+	M2	3	53	89	70	myeloid
25.	+++	+++	+	+++	+++	M4	5	69	nt.	47	myeloid
26.	–	–	–	–	+	M0	13	21	nt.	67	myeloid
27.	–	–	–	–	+	M0	1	1	85	32	myeloid
28.	++	++	+	++	+–	M1	32	28	62	49	myeloid
29.	++	++	+	++	+	M2	2	28	40	27	myeloid
30.	++	++	+	++	+, ++	M4	24	3	25	25	myeloid
31.	++	+, ++	++	nt.	++, +	M4	5	16	10	28	myeloid

nt. = not tested

Results

FAB classification: following the criteria of the FAB group, 56 patients were classified. 27 patients belonged to myeloid type: MO: 8, M1: 3, M2: 8, M4: 12, M5: 1, and 25 cases were of lymphoid origin: L1: 4, L2: 20, L3: 1.

The cytochemical reactions have made the differential diagnosis of the blast cell more accurate. Within the lymphoid cells the PAS positivity is characteristic of the B-lymphoblast, while the AP reaction serves the differential diagnosis of T-lymphoblasts. Myeloid and lymphoid cells could be differentiated from each other with the help of DN-ase, MPO and Sudan BB reactions. Over 70% of the myeloid leukaemia cases showed positive reactions by MPO, Sudan BB and NASDCA stains, while DN-ase reaction was positive in 16/31 cases (51.6%) (Tables 1 and 2).

The ANAE reaction was positive in 23 out of 31 cases (74.1%) and dubious in 4 cases. The reaction was found to be strongest in the case of M4 leukaemias. The cellular muramidase reaction was performed to prove the monocytoid characteristics as a supplement of the other reactions.

Immunological characterisation of the myeloid leukaemias: With the help of the monoclonal antibody panel used in our laboratory we confirmed the myeloid characteristics in 28 cases of leukaemias. In three of the patients the HLA-DR antigen was the only marker that could be detected on the cell surface (case 8, 18, 19). In these cases the B-cell characteristics could be excluded on the basis of a negative intracytoplasmic CD22 determination, thus we thought this leukaemia to be of stem cell origin. We tried to compare the results of the FAB classification and of immunological marker studies (Table 3). CD13, DR, CD33 antigens were present in all of the M1 cases. In the M2 subgroup the leukaemic cells expressed CD13, CD33 and HLA-DR antigens, while two cases were found to be CD14 positive, too. A variable presence of CD14, CD13, CD33 and HLA-DR recognized antigens was observed in AML subtype M4. The one patient with M5 subtype was characterised as a CD14, CD13, CD33 and HLA-DR positive case. All M0 type AML were CD14 negative and HLA-DR positive, while myeloid specific antigen (CD13 or CD33) was found on the cells in five cases.

Table 3

Comparison of FAB subclasses and positivity of monoclonal antibodies in AML patients

Monoclonals	Numbers of Patients				
	M0 : 8	M1 : 3	M2 : 8	M4 : 12	M5 : 1
CR14+	0/8	0/3	2/8	5/12	1/1
CR13+	3/8	3/3	7/7	9/11	1/1
CR33+	2/8	3/3	5/6	6/9	1/1
HLA-DR+	8/8	2/3	7/8	9/12	1/1

Cytochemical and immunological data of *ALL patients* are shown in Tables 4 and 5. Using cytochemical stain, 7 cases marked as ALL B-cell type, and 9 cases as acute undifferentiated leukaemia (AUL) (Table 4). Six non-T ALL cases were only HLA-DR positive by immunophenotyping (group-I = stem cell leukaemia). In these cases the intracytoplasmic CD3 and CD22 also proved to be negative. HLA-DR, CD19 and CD10 antigens were simultaneously present in four cases, these were classified as group III. Group VI. represents the most mature type of non-T ALL. In these four cases the cells expressed surface immunoglobulin (sIg) and other antigens, including CD20, CD19, and HLA-DR. CALLA antigen (CD10) may also be present (Table 4). The immunophenotype of the leukaemic cells was questionable in two cases (No. 32, No. 36). The gene rearrangement was not tested in one of these two patients and it was germ line in the other case. The immunodiagnosis was confirmed by Ig heavy chain gene rearrangement in ten non-T ALL cases. In one case (No. 34) the results of immunophenotyping and gene rearrangement study were contradictory.

The simultaneous positivity of the AP and negativity of Sudan BB reactions were used as cytochemical markes in T-cell ALL (Table 5). Using these criteria 9 cases were found to be T-cell by cytochemistry, while only 7 of these cases proved to be T-ALL by immunophenotyping. One of the two latter cases (No. 50) was a non-T ALL-III type (shown CD10+ and HLA-DR+ as well as CD2−, CD3−, CD8−, CD4−), while the other patient (No. 51) was diagnosed as having AML (CD13+). The criteria of Foon and Todd [8] were used for the evaluation of the T-ALL case, too. CD3, CD2, CD4, CD8 and CD1 antigens were detected on the surface of cells in 3 cases (group-II), and CD3, CD2, CD4, CD8 positivity and CD1 negativity was found in 2 cases (group-III). In two cases (No. 48, No. 53) the subgroups were undeterminable. The origin of the leukaemic proliferation was proved by TCR beta chain gene rearrangement in the 5 thus investigated T-ALL cases.

The purpose of this study was to compare the morphological, cytochemical and immunological characteristics of acute leukaemias. It is important to distinguish lymphoid from myeloid blast cells from the point of view of prognosis and therapy.

Morphological and cytochemical studies do not lead to the correct diagnosis in all cases. We used four monoclonal antibodies described to be of myeloid lineage in our AML cases (Tables 1, 2, 3). In our patients this panel of antibodies provided no more information than the cytochemical stains. Drexler et al. [5] using a broader monoclonal antibody panel found a correlation between the expression of the cell surface markers and the FAB subgroups, but immunologically only 3 subgroups could be established. Even among these groups there was a correlation only between the immature cells FAB M1 and the immunological phenotype (group I, HLA-DR+, CD33+). The reason for this is not only that these diseases are extremely heterogenous [8, 24, 25], but also that myeloid reactive antibodies may react with subsets or differentiation stages of lymphoid

Table 4

Results of patients with ALL non-T-cell type (n = 16)

Patients	FAB	PAS	Cytochemical Classification	CD19 %	CD20 %	CD21 %	HLA-DR %	CD10 %	sIgM %	Immunol. Phenotype	Genotype
32.	L2	+	ALL-B?	20	26	12	nt.	nt.	3	?	nt.
33.	L2	+	ALL-B?	14	4	2	62	16	13	B-I.	nt.
34.	L2	++	ALL-B.	87	89	5	88	41	84	B-VI.	T
35.	L2	+	ALL-B?	7	1	1	52	14	15	B-I.	B
36.	L2	–	AUL	4	3	0	nt.	0	10	?	G
37.	L2	–	AUL	nt.	nt.	nt.	75	92	5	B-III.	nt.
38.	L2	–	AUL	54	6	10	82	73	8	B-III.	B
39.	L3	–	AUL	4	4	3	74	4	20	B-I.	G
40.	L2	–	AUL	9	nt.	nt.	55	15	nt.	B-I.?	B
41.	L2	–	AUL	21	70	9	80	27	73	B-VI.	B
42.	L1	+	ALL-B?	12	9	14	31	41	30	B-I.	B
43.	L2	–	AUL	63	37	30	83	80	80	B-VI.	B
44.	L1	–	AUL	74	55	nt.	72	13	97	B-VI.	B
45.	L2	–	AUL	96	94	nt.	84	90	3	B-III.	B
46.	L2	+	ALL-B?	4	8	6	54	21	24	B-I.	B
47.	L2	+	ALL-B?	45	nt.	nt.	49	42	8	B-III.	B

nt = not tested; AUL = acute undifferenciated leukaemia; G = germ line

Table 5

Results of patients with ALL T-cell type (n = 9)

Patients	FAB	Acid pH	CD3 %	CD2 %	CD4 %	CD8 %	CD1 %	CD10 %	HLA-DR %	Immunol. Phenotype	Genotype
48.	L2	+, −	60	80	nt.	nt.	nt.	nt.	12	T-ALL**	T
49.	L1	+, −	nt.	92	65	67	61	nt.	nt.	T-ALL-II.	T
50.	L2	+	10	20	5	3	nt.	42	85	non-T-ALL	nt.
51.	L2	+	2	2	nt.	nt.	nt.	10	20	Myeloid My 7 : 72%	germ line
52.	L2	+	66	92	35	24	6	3	4	T-ALL-III.	T
53.	L1	+	73	72	12	7	nt.	11	16	T-ALL**	nt.
54.	L2	+, −	nt.	63	55	70	62	11	13	T-ALL-II.	T
55.	L2	+	49	92	37	28	0	3	6	T-ALL-III?	nt.
56.	L2	+	91	87	89	81	91	0	3	T-ALL-II.	T

nt = not tested ** = intracytoplasmic CD3 was positive

cells [7, 9, 10, 17]. Moreover, many myeloid antigens are not or only partially expressed on immature cells. Recently, the best immunological reagent for the recognition of AML seems to be a monoclonal antimyeloperoxidase antibody [26]. Reactivity with this antibody certainly seems to be a better test for AML than standard cytochemistry and has the advantage of making quantification possible.

In ALL the cytochemistry could not exactly separate T- from B-lineage [27]. The staining patterns of AP (Table 5) of immature T-cells is not always characteristic. The PAS positivity of non-T lymphoid cells (Table 4) is also uncertain. In those patients who had non-T ALL according to FAB criteria, the panel of monoclonals CD19, CD20, CD21, CD10, HLA-DR helped us to provide a more accurate diagnosis. Immunophenotyping made it possible to divide the cases into prognostically important subgroups. The immunological study provided valuable additional information expecially in our AUL cases (Table 4). We found a very good correspondence between the results of immunophenotyping and Ig gene rearrangement in non-T ALL. Results of immunophenotyping and DNA study were contradictory only in one case (No. 34). This patient was found to have B-ALL, group VI, while the TCR beta chain gene was rearranged (RG/GG). The Ig heavy chain gene was in germ line configuration. The results of immunological investigation and T-cell receptor gene rearrangement in ALL of T-cell type are in agreement in all tested cases (Table 5).

In problematic ALL cases, intracytoplasmic CD3 and CD22 staining are of diagnostic value. These early T and B cell markers may help to discriminate between immature (thymic and bone marrow) and mature T and B cell leukaemias/lymphomas [28].

In conclusion: immunophenotyping based on the use of monoclonal antibodies makes the objective and reproducible differentiation of acute lymphoid and myeloid leukaemias as well as lymphoid leukaemias of T and B origin possible. Based on our results it can be stated that immunophenotyping did not help in distinguishing the subtypes of AML. Cytochemical methods can be well applied for purposes of clinical diagnostics. On the other hand, the diagnosis of acute lymphoid leukaemias is not possible without use of monoclonal antibodies.

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Hairy Cell Leukemia. Immunological Study

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The study has been performed on peripheral blood and splenic malignant cells from 16 patients with hairy cell leukaemia (HCL). The cell surface markers were identified by rosette techniques and using monoclonal antibodies (m Ab). The surface markers' expression of the hairy cells (HC) varied. The E receptors, the T-cell antigens, the HLA-DR antigens and smIgG were either expressed or not according to the affected organ, the progress of illness, or the treatment. The surface pattern changed sometimes in the same patient during the progress of illness. These observations demonstrate that HCL is a unique disease with malignant cells characterized by a marked variability of the cell surface markers.

To demonstrate the ability of hairy cells to bind labile smIgG, the cells were studied by affinity chromatography on SpA-Sepharose 6MB and by ES-rosette assay. The percent of cells bound on SpA-Sepharose varied between 6% and 66%, representing the hairy cells with labile-bound smIgG. With affinity chromatography it was also possible to separate the hairy cells with a special phenotype: T_3^+ T_4^+ T_8^+ T_{11}^+ surface membrane labile-bound IgG^+ ($lIgG^+$) FcR^+, $HLA\text{-}DR^+$ $EACD^+$ (Ripley rosette forming cells), resembling a normal subset of large granular lymphocytes (LGL). The percentage of these cells varied between 60% and 86% of the bound cells. These observations suggest that in HCL, the malignant transformation might involve a common progenitor for the B, T and LGL lineages, the hairy cell being a hybrid type of malignant cell. Its main immunological peculiarity is the marked mobility of the surface membrane structures and hence the lability (plasticity) of the surface markers' expression.

Keywords: affinity chromatography, hairy cell, pathogeny, surface markers

Introduction

It is generally admitted that the hairy cell represents a special type of malignant B-cell [1, 2, 3], although it often displays some features which are characteristic for T-cells [4], or macrophages. Recent data [5, 6] have shown that sometimes hairy cells express simultaneously markers characteristic for different cell-lineages. Therefore, it was suggested that in hairy cell leukemia the malignant disturbance induces the appearance of a hybrid neoplastic line [6]. The following communication presents data in support of this hypothesis. Sixteen patients admitted to the Clinic of Haematology in the last 5 years were investigated. The malignant cells were studied using monoclonal antibodies, rosette-tests and affinity chromatography on SpA-Sepharose 6 MB. The effect of splenectomy and/or chemotherapy upon the hairy cell surface markers is also discussed.

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Materials and Methods

Cells.

Mononuclear cells were purified from the peripheral blood and/or spleen of 16 patients by sodium metrizoat-Ficoll centrifugation [7]. The mononuclear cells were again separated by centrifugation over dense Ficoll-Hypaque (specific gravity 1084). The cells at the interface represented the fraction enriched in HC. In all experiments, the Ficoll preparations were stained to exclude granulocyte contamination. Hairy cell leukaemia is characterized by an almost complete monocytopenia and by a deficiency of circulating normal B-cells [8]. The purity of the HC fraction varied between 65% and 99% (99% in three cases with high percent of HC) in the peripheral blood and between 80% and 90% in the spleen, as confirmed by the presence of acid phosphatase resistant to tartaric acid (TRAP). The viability of cells was never below 95% as demonstrated by the trypan blue method.

Monoclonal antibodies (m Ab)

Purified m Ab preparations (belonging to the IgG_1 isotype) directed against T_3, T_4, T_8, T_{11} and HLA-DR lymphocyte membrane antigens, were kindly supplied by Dr. Chang Yi Wang (Memorial Sloan Kettering Cancer Center, New York, NY).

Staphylococcus protein (SpA) coated erythrocytes (ES reagent)

Bovine erythrocytes were coated with SpA as previously described [9].

ES-rosettes assay

Monoclonal antibodies were used in an ES-rosette assay as already described [10]. Briefly, the suspension of cells in culture medium (2.5×10^5) is incubated for 30 min in ice with the respective monoclonal antibodies (0.005 ml). After three washings in cold culture medium, the cells are suspended in 0.2 ml culture medium and treated with 0.015 ml ES reagent. The mixtures are centrifuged at $100g$ for 10 min and incubated for 2 hr or overnight at 4 °C. The pellet is gently resuspended and treated with 0.030 ml of 0.2% toluidine blue in phosphate buffered saline.

Assay for labile-bound smIgG

Freshly prepared peripherial blood or splenic hairy cells (10^6/0.5 ml) were mixed with ES reagent and centrifuged at 4 °C for 10 min at $100g$. The samples were stored at 4 °C overnight. The cells preincubated at 37 °C for 2 hr were washed with culture medium and rosetted with ES-reagent in the same conditions.

EACD (Ripely) – rosette assay

Freshly prepared cells (10^6/0.5 ml) were mixed with EACD reagent (human erythrocytes group 0 Rh-positive, sensitised with human anti CD serum 1 : 16,000). After centrifugation at 4 °C for 10 min at 100*g*, the samples were stored for 30 min at room temperature, or overnight at 4 °C.

The HC nature of the rosette forming cells (E, ES, EACD and m Ab-ES-rosettes) was identified on the Romanowski stained preparations and by the presence of TRAP.

Affinity chromatography on SpA-Sepharose 6MB

SpA-Sepharose 6MB was prepared as previously described [10], and could bind 4 mg IgG/ml of packed gel. Cells were fractionated on SpA-Sepharose 6MB as previously described [11]. Briefly, about 5×10^7 cells suspended in 0.5 ml culture medium (RPMI containing 1% NaN_3 and 0.5% from a solution of 10% gelatine in P.B.S.) were loaded on SpA-Sepharose 6MB in a small size plastic column (1 × 5 cm) and incubated for 45 min at 4 °C. Unbound-cells were washed from the column with 25 ml of cold RPMI medium. The specifically-bound cells (with IgG on the surface) were eluted mechanically by adding 5 ml of RPMI medium warmed at 37 °C, and resuspending the gel on the upper part of the column. The procedure was repeated twice, and the eluted cells were collected by centrifugation. The unbound-cells as well as those retained on the column and eluted by resuspension were investigated for cell surface markers. The hairy cell nature of the rosette forming cells was identified on the Romanowski stained preparations and by the presence of TRAP.

TRAP activity was identified using the Li Cy, Yam Ly technique [12] and recently according to the technique described by Takashi Machii [13].

Transmission electron microscopy (TEM) was performed on 1% osmium tetroxide – fixed PBMC (1 hr at 4 °C). The cells were then dehydrated and embedded in Epon 812. Thin sections cut with an ultramicrotome were stained with uranyl acetate and lead citrate [14].

Results

The diagnosis of HCL was made on the basis of the clinical picture, characteristic cytology of the abnormal cells from the peripheral blood, from the bone marrow biopsy (performed in all cases) and from the spleen. The histopathological preparations from the bone marrow and spleen revealed the special disposition of HC. The percentages of HC were established by phase contrast microscopy and confirmed by the presence of acid phosphatase resistant to tartaric acid (TRAP) in all cases. The almost total replacement of splenic tissue by HC was confirmed by TRAP reaction. The transmission electron microscopy of the peripheral blood mononuclear cells was performed in all cases and showed morphologically typical HC, a proportion of which contained ribosome-lamellar complexes (myelinoid bodies).

Table 1

Clinical and laboratory data of the HCL patients

No.	Name	Sex	Age (yrs)	WBC count/mm³	Nr/mm³ and of HC	%	Liver en-largement cm	Spleen enlarge-ment, sm	Treatment	Progress of disease	Cause of death
1.	SN	♂	50	1,600	520	(32)	1	6	Corticotherapy Splenectomy	72 months	—
2.	CG	♂	53	5,200	3,200	(67)	3	10	Corticotherapy Splenectomy	36 months	—
3.	SA	♂	46	3,900	1,100	(30)	1	1	lithium carbonate Corticotherapy	AIHA 36 months	sepsis
4.	CN	♂	59	4,600	2,150	(46)	10	12	lithium carbonate Corticotherapy	4 months	acute fi- brynolysis
5.	DR	♂	59	16,200	15,400	(96)	1	3	CHOP	progressive disease (fever, lymphnode enlargement) 18 months	sepsis
6.	DZ	♂	43	5,400	4,200	(78)	1	4	CHOP Corticotherapy	progressive disease 12 months	sepsis
7.	CD	♀	53	1,200	750	(62)	2	4	Corticotherapy	AIHA broncho- pulmonary infec- tions 2 months	sepsis
8.	PV	♂	28	3,400	1,800	(53)	1	4	Splenectomy	pulmonary tuber- culosis treated and cured 44 months	—

9.	GI	♂	74	14,000	11,200	(80)	2	5	CVP Corticotherapy	14 months	heart failure
10.	IL	♀	49	2,200	1,200	(54)	10	15	Splenectomy	pulmonary tuber-culosis 2 months	sepsis
11.	MI	♂	58	1,200	480	(50)	1	4	Splenectomy	6 months	sepsis
12.	ME	♂	35	1,600	350	(22)	3	2	Splenectomy	19 months	—
13.	TD	♂	68	2,600	1,450	(56)	1	3	Corticotherapy Splenectomy	36 months	—
14.	RC	♂	52	2,100	840	(40)	10	3	lithium carbonate Corticotherapy Splenectomy	hepatic cirrhosis 2 months	acute hepatic failure
15.	IV	♂		16,000	13,600	(85)	1	10	Corticotherapy Splenectomy	14 months	—
16.	SN	♂	48	4,500	2,475	(55)	2	4	Corticotherapy Splenectomy	36 months	—

HC = hairy cells
AIHA = autoimmune haemolytic anemia
WBC = white blood cells

Table 1 demonstrates some clinical and laboratory data of our patients at admission. Most of the patients (87%) were males of middle age. Only three patients (19%) had a leukemic spread, but in most of the cases a high proportion of the WBC were HC. The haemoglobin varied between 4.5 g% and 14 g%, the lowest values were observed in two cases of autoimmune haemolytic anemia (AIHA). The platelet count varied between 20,000 and 120,000. All of the patients had liver and spleen enlargement. Forty-three percent of the patients are still alive; the cause of death was sepsis in the majority of the cases.

Table 2

Surface markers of hairy cells*

Surface markers	Specificity	Number and percent of cases	Percent of positive cells
T_3 (CD_3)	Pan T mature cells	9 (56.2)	18–86
T_4 (CD_4)	helper T cells	7 (53.8)	23–77
T_8 (CD_8)	cytotoxic/suppressor T cells	9 (56.2)	17–79
T_{11} (CD_2)	sheep erythrocyte receptor	9 (56.2)	20–69
HLA-DR	B-cells, monocytes, activated T-cells, leukemic cells	16 (100)	12–84
smIgG	surface membrane stable bound IgG	11 (68.7)	12–52
$_{l}$IgG	surface membrane labile-bound IgG	10 (62.5)	6–81
FcR	receptors binding IgG (EA-positive cells)	16 (100)	60–82
FcγR	receptors binding human IgG (EACD-positive cells)	14 (87.5)	0–65

* Splenic and/or peripheral blood hairy cells

Table 2 gives the results with surface markers of peripheral blood and splenic hairy cells. It can be seen that HLA-DR antigens and Fc receptors were expressed by HC in all cases, the percentages of positive cells varying between 12–84% and 60–82%, respectively. In most of the patients, the HC were also EACD-positive. The T-cell antigens were expressed in over 50% of the cases. Only about 31% of cases were E negative and 25% were smIgG negative. The HC presented a large variability regarding the expression of surface markers. The E receptors, the T-cell antigens, the HLA-DR antigens and smIgG were expressed or not according to the affected organ, the course of illness or the treatment. Sometimes the surface pattern changed in the same patient during the course of the disease.

Affinity chromatography on SpA-Sepharose 6MB and ES-rosettes assay demonstrated the ability of HC to bind labile smIgG. The percent of cells bound on SpA-Sepharose – representing the HC carrying mainly labile-bound IgG – varied between 6% and 66%.

Table 3

Surface markers of peripheral blood and splenic hairy cells separated by affinity chromatography on SpA-Sepharose 6MB

Case No.	Markers	Peripheral blood			Spleen			Peripheral blood		
		Initial cells	Bound cells	Unbound cells	Initial cells	Bound cells	Unbound cells	Initial cells	Bound cells	Unbound cells
1*	T_3^+	55	81	20	62	86	14	55	67	29
	T_4^+	40	50	10	47	51	12	45	65	27
	T_8^+	55	60	9	70	81	9	55	67	0
	T_{11}^+	50	62	8	58	67	2	52	60	20
	DR^+	29	37	28	19	29	18	72	84	59
	EA^+	72	73	74	80	81	82	79	72	73
	$EACD^+$	22	36	1	25	28	2	12	20	1
	$smIgG^+$	46	6	NT	30	8	2	44	12	4

* 5×10^7 cells/0.5 ml were introduced in the column and 66% were bound on SpA-Sepharose

NT — not tested

Table 4

Surface markers of peripheral blood hairy cells separated by affinity chromatography on SpA-Sepharose 6MB

Case No.	Markers	Initial cells	Bound cells	Unbound cells
2*	T_3^+	25	30	10
	T_4^+	9	1	8
	T_8^+	7	20	0
	T_{11}^+	0	0	0
	DR^+	20	44	3
	EA^+	78	85	70
	$EACD^+$	18	24	1
	$smIgG^+$	34	84	0
3**	T_3^+	39	57	28
	T_4^+	20	13	33
	T_8^+	51	56	2
	T_{11}^+	60	67	10
	DR^+	35	36	33
	EA^+	80	85	70
	$EACD^+$	0	0	0
	$smIgG^+$	50	49	10

* 5×10^7 cells/0.5 ml medium were introduced in the column and 6% were bound on SpA-Sepharose

** 5×10^7 cells/0.5 ml medium were introduced in the column and 55% were bound on SpA-Sepharose

In Table 3, the surface markers of peripheral blood HC (before and after splenectomy) and splenic HC separated by affinity chromatography on SpA-Sepharose 6MB are shown. The percentages of cells expressing T-cell markers and EACD-positive cells are much diminished in the unbound population (especially the T_3, T_8 and T_{11}-positive cells). After splenectomy, the percentages of HLA-DR positive cells in all the three columns are much increased. Only a little part of the bound cells were ES-positive and represented the cells which had maintained the IgG molecule on their surface during the cell-release from the SpA-Sepharose.

Table 4 gives the results of surface markers of peripheral blood HC separated by affinity chromatography in two patients. Six percent and 55%, respectively of the cells introduced to the column were bound on SpA-Sepharose. The cell population bound on SpA-Sepharose was rich in HC positive for T_3, T_8 antigens and smIgG in both cases. In case No. 2 the HC were also EACD-positive. The HC bound on SpA-Sepharose expressed a special phenotype: T_3^+ T_4^+ T_8^+ T_{11}^+ $_1IgG^+$ Fc^+ DR^+ $EACD^\pm$. The percent of cells with such a phenotype varied between 66% and 86% of the bound cells. The unbound HC had stable-bound IgG, presented lower percentages of T-cell antigens and were EACD-negative.

Table 5

Phenotypical changes induced by splenectomy

1 Appearance of new markers	1 case
2 Appearance of some antigens in a higher percentage	4 cases
3 Transient expression of some antigens	3 cases
4 Maintainance of the same phenotype	1 case
5 Disappearance of some markers	1 case

Table 5 presents the phenotypical changes induced by splenectomy. In most of the cases (88.8%), splenectomy modified the surface phenotype by the appearance or disappearance of some surface markers by the expression of some antigens in a higher percentage, or by the transient expression of some antigens.

Discussion

The presence of the labile-bound smIgG – as demonstrated by affinity chromatography and ES-rosette assay – and the ability of some hairy cells to form EACD (Ripley) rosettes are characteristic for the LGL lineage. By affinity chromatography on SpA-Sepharose 6MB, a cell-population enriched in hairy cells with a particular phenotypic pattern was separated: T_3^+ T_4^+ T_8^+ T_{11}^+ $_1IgG^+$ Fc^+ $R^\pm$ $DR^\pm$. This phenotype resembles those of a subset of normal LGL sepa-

rated by affinity chromatography from the normal peripheral blood lymphocytes and normal splenocytes.

Recent data [15] have shown that the LGL with T_3, T_8 (or T_4) and T_{11} antigens represent the cytotoxic T lymphocytes (CTL) which are sometimes FcR-positive. The latter feature explains the CTL binding on SpA-Sepharose. The few T_4-positive cells among LGL population might represent either the NK subpopulation with T helper phenotype [16] or the CTL-T_4 positive, MHC class II restricted [15]. However, the question regarding the relationship between these LGL subpopulations and HC remains open, further investigations are necessary.

It is known that all of the hairy cells – as well as LGL cells – express T_{ac} receptors spontaneously. The T cells express these antigens only after the antigenic stimulation. It was also demonstrated that hairy cells have the ability of changing their phenotype in cultures with PHA. In these conditions the $E^- T^- IgG^+ BA_1^+$ cells became $E^+ T^+ IgG^- BA_1^-$ [17]. Our study showed that this phenomenon takes place also "in vivo", after splenectomy.

Our observation demonstrated that the malignant LGLs display – in phase-contrast microscopy – similar characterics as hairy cells. In agreement with other workers [13] some of our unpublished observations have shown that leukemic LGL are positive for tartrate resistant acid phosphatase (TRAP). Our observation that some normal LGL present unicentric myelinoid bodies similar to those observed in hairy cells might be correlated to some recent data [18] which report that a subpopulation of LGL show a selective phagocytosis of Gram-positive bacteria, similarly to myelomonocytes.

These observations suggest that in hairy cell leukemia the malignant transformation involves a common progenitor for B cell, T cell and possibly for the LGL lineage. The phenotypical variants and their lability – the main immunological peculiarity of hairy cells – might be associated to the extreme mobility of the surface membrane structures, demonstrated by the fast capping phenomenon. In agreement with other authors [6] we believe that in hairy cell leukaemia the malignant disturbance induces the appearance of a hybrid neoplastic line, or that the disease is linked to a major disturbance in stem cell commitment. The hybrid types of malignant cells have also been described in leukaemias and malignant lymphomas, in which the malignant transformation involves a progenitor cell that may possess multiple maturational capacity [19, 20, 21].

In conclusion, the results of the present study show that the hairy cell is a hybrid type of malignant cell. The main immunological peculiarity of this cell is the marked mobility of the surface membrane structures, and hence the lability (plasticity) of its immunophenotype. The surface pattern changed sometimes in the same patient, the surface markers were either expressed or not, according to the affected organ, the progress of the illness or the treatment, thus demonstrating that HCL is an unique disease with malignant cells characterized by a marked variability of the surface antigenic structures.

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Distribution and Scatter of Yeast Cell Phagocytosis by Human Monocytes in an Improved Glass Surface Assay

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The assessment of yeast cell phagocytosis by glass-adherent monocytes was improved by ultrasonication of yeast cells prior to the experiments in order to prevent aggregation, restriction of measurements to completed engulfment regardless of the number of partides ingested, and stopping of phagocytosis before counting by maintained cooling and the addition of EDTA. The modifications provided a reduced dispersion of individual values, increased engulfment of yeast cells, and decreased numbers of yeast cells only adherent to the monocyte membrane. The improvement made the method more convenient for the study of engulfment by monocytes.

Keywords: monocytes, yeast cells, phagocytosis

Introduction

The Hed technique of studying yeast cell phagocytosis by glass-adherent neutrophils [1] was modified by Athlin and co-workers and found useful for the study of the effects of some antineoplastic agents on monocyte phagocytosis, both on the adherence step and the engulfment step [2]. The scatter of individual values was, however, inconveniently wide and skew [3].

The aim of the present study was to improve assessment of yeast cell phagocytosis by glass-adherent monocytes. The study was restricted to the engulfment stage of phagocytosis. The modifications introduced consisted of ultrasonification of the yeast cells in order to reduce aggregation, counting of engulfment as an all-or-none process in the individual monocyte (fraction of monocytes with at least one ingested yeast cell), maintenance of ice-cool temperature by keeping the samples on an ice bed during counting, and addition of 1 mM EDTA to the cold stopping of the phagocytosis process [4, 5].

Materials and Methods

Blood sampling

Heparinized blood, 16 IU/ml was obtained from blood donors at the Blood Centre of the hospital as in previous studies [2, 3]. The processing of the sample was started within one hour and finished within 6 hours after blood collection.

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Isolation of mononuclear leukocytes

Mononuclear leukocytes (MNLs) were isolated from peripherial blood by the one-step metriozate-Ficoll procedure, using Lymphoprep® (Nycomed, Oslo, Norway). The MNLs were then washed twice in phosphate-buffered saline (PBS) and resuspended in "complete medium" to a concentration of 5×10^6 cells/ml.

Incubation for monocyte adherence to the glass surface

MNLs, 1×10^6 cells in complete medium, were then seeded on sterile slides in Petri dishes and incubated in a humified chamber with 5% CO_2 at + 37 °C to allow the monocytes to adhere to the glass surface. For the removal of lymphocytes from the preparation, the slides were rinsed with pre-warmed complete medium at +37 °C. The remaining cells were stained for non-specific esterase, (data not shown).

Preparation of yeast cell suspension

Baker's yeast (Saccharomyces cerevisiae) was suspended in saline, washed twice, boiled 30 min, washed twice again, and labelled with fluorescein isothiocyanate, FITC (Merck, Darmstadt, FRG) in carbonate buffer, pH 10.2, 10^8 cells per ml of FITC buffer with a FITC concentration of 0.1 mg/ml for 60 min at +37 °C. The cells were washed three times with saline, re-diluted to 10^8 cells per ml, and frozen at −17 °C in 0.5 ml aliquots. Before the experiment, the FITC-labelled yeast cells were opsonized with autologous serum (0.5 ml mixed with 0.5 ml of the FITC-labelled yeast cells), incubated at +37 °C for 30 min. The cell suspension was then washed twice and resuspended in 2 ml of saline. Ultrasonication was performed in a continuous cycle for one minute to prevent yeast cell aggregation (Branson Sonofier, Cell Disruptor B15).

Phagocytosis experiments

Opsonized yeast cells labelled with FITC, 5×10^6 yeast cells in 200 μl saline were added to the slides and incubated for 30 min at +37 °C in 5% CO_2. The ratio of effector/target cells was $0.5-1.0 \times 10^2$. The preparation of test samples (n = 38) was modified compared to that of previous control (n = 304) by ultrasonication of yeast cells to prevent aggregation. Phagocytosis was stopped by dipping the slides in ice-cold PBS, pH 7.4. In the test samples, ice-cold temperature was maintained during the counting procedure by the use of an ice bed. The stopping of phagocytosis in the test samples was further improved by addition of EDTA, 1 mM (5). The fluorescence of non-ingested yeast cells was then quenched by dripping trypan blue in saline (Sigma, St. Louis, Mo, USA), 0.5 mg/ml, onto the slides. The staining time was 10 s. The stain was decanted and the preparation was sealed with a coverslip. The fractions of monocytes without associated yeast cells, with only adherent yeast cell(s) and engulfed yeast cell(s)

were counted in 50 consecutive monocytes at 320 × magnification with a Zeiss incident-light microscope.

Complete medium

The complete medium consisted of 165 ml heat-inactivated human serum, 7 ml L-glutamine 20 mM, 500 ml RPMI 1640 medium (Flow Laboratories, Irvine, Ayershire, Scotland), and benzylpenicillin 2.4 μg/ml.

Glassware

The glassware was thoroughly washed, rinsed in demineralized water and 70% ethanol, and autoclaved at +120 °C for 20 min.

Statistics

Descriptive and analytical statistics (a two-tailed t-test) were performed by StatWorks™ (Heyden Son, London).

Results

It is evident from Figure 1A that ultrasonication of the yeast cell suspension, carefull control of the temperature after "stopping" of phagocytosis, and addition of EDTA to the steady-state suspension of cells improved the assessment of engulfment, the present marker of phagocytosis, from a median value of 65% monocytes with at least one ingested yeast cell to 90% ($p < 0.001$). The improvement was associated with a conspicuous decrease in the scatter of individual values. However, the distribution still had a minor left skew (Fig. 1A).

Corresponding to the improvement of engulfment (Fig. 1A), the yeast-to-monocyte adherence decreased ($p < 0.001$) with a concommittant decrease in dispersion of individual values. However, the adherence dispersion still had a minor right skew (Fig. 1B). The changes depicted in Figure 1 (A and B) thus indicate a shift from phagocytosis recorded in the adherence step to phagocytosis recorded in the stage of performed engulfment.

The influence of cold shock with and without maintenance of ice-cool temperature was then studied (Fig. 2). Intermittent cooling with return to room temperature reduced engulfment to 27% of control values. When ice-cool temperature was maintained, engulfment was reduced to 15% of control values.

Addition of EDTA, 1 mM, reduced phagocytosis to 3%. Since EDTA by itself reduced phagocytosis to approximately 20% of control values (not shown), the effects of EDTA and maintained cooling were additive and virtually abolished engulfment after the first minute of control conditions (Fig. 2).

Discussion

Phagocytosis is a labile series of events, which usually requires the processing of control and test samples from the same donor simultaneously [2, 3]. Present data are such control values from different experiment series, in which basic conditions were kept constant. The apparent improvement in the present test technique [4, 5] made a comparison with our previous controls feasible and unbiased.

The precision of phagocytosis assessment in glass-adherent monocytes was increased by the modifications introduced in the present study (Fig. 1). It is reasonable to assume that meticulous stopping of yeast cell phagocytosis during counting after the incubation time – maintained cooling and addition of EDTA – was the essential modification. Each step alone suppressed engulfment down to 20% of control values [5]. The combined effect was additive and virtually abolished further engulfment (Fig. 2).

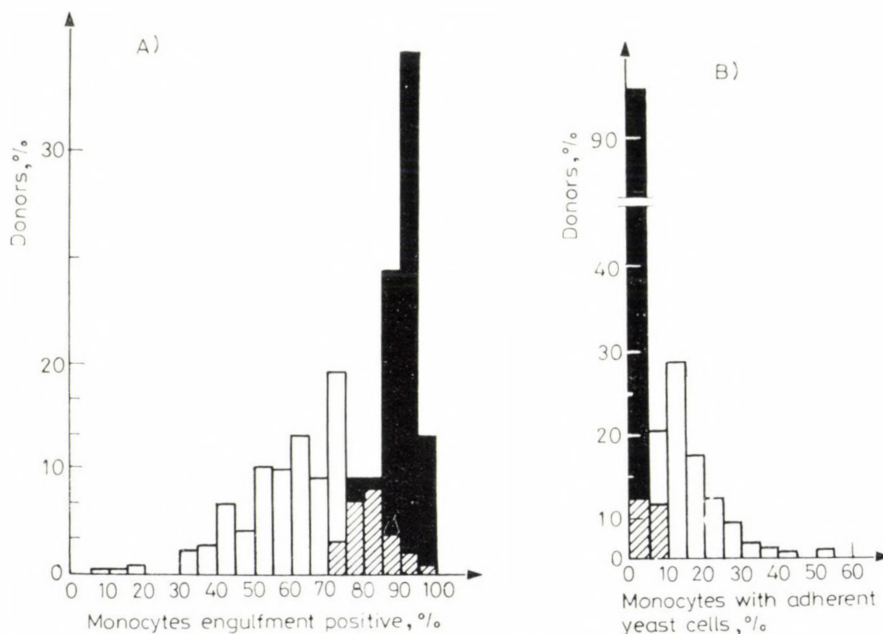

Fig. 1A. The fraction of monocytes containing ingested yeast cells in our previous controls (white, n = 304) and in test samples (dark, n = 38). Shaded area shows overlapping. The distribution of values our previous controls was characterized by median 65%, interquartile range 54–74%, range 6–96%, skewness –0.568, kurtosis 0.500, test samples by median 90%, interquartile range 86–94%, range 72–100%, skewness –0.537, kurtosis –0.445

Fig. 1B. The fraction of monocytes with only adherent yeast cell in our previous controls (white, n = 304) and in test samples (dark, n = 38). Shaded area shows overlapping. The distribution of values in our previous controls was characterized by median 12%, interquartile range 6–18%, range 2–52%, skewness 1.467, kurtosis 2.705, test samples by median 2%, interquartile range 2–4%, range 0–8%, skewness 0.626, kurtosis –0.502

Monocyte phagocytosis in vivo is a complex variable which involves adherence to a surface, locomotion, adherence between phagocyte and particle engulfment, processing, and secretion of degradation products [6, 7, 8]. One attractive aspect of the Hed technique is that phagocytosis is measured in surface-adherent phagocytes with discrimination of phagocyte-to-particle adherence and the subsequent step, complete engulfment of the particle. However, restriction to completed engulfment make repeated measurements more convenient and retain most of the information [2].

The dispersion of individual values in the measurement of monocyte function is a widely recognized phenomenon [9]. It is reasonable to assume that the dispersion reflects heterogeneity in the studied monocyte population as regards maturation and differentiation. Although diversity is as fine as dispersion is foul, the phenomena may represent two sides of the same coin. Nevertheless, the present restrictions and modifications of assessing monocyte phagocytosis make the technique more precise and convenient.

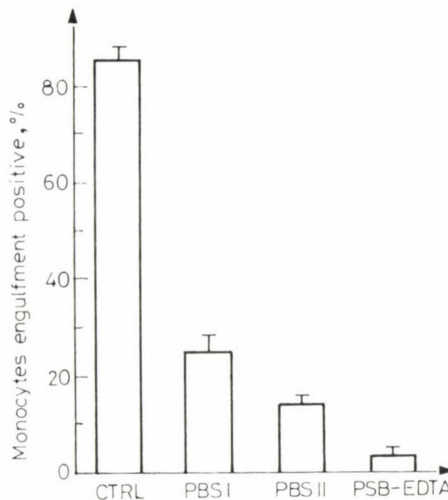

Fig. 2. The control monocytes (CTRL) were allowed to ingest yeast cells for 30 minutes. The reaction was then stopped by dipping the slides in ice-cold phosphate-buffered saline (PBS), 2 ml, containing 1 mM EDTA. Continuing of phagocytosis during temperature equilibrium with room temperature was then studied by addition of yeast cells to glass-adherent monocytes for one minute, adding 2 ml ice-cold PBS to the Petri dish, and then incubated at room temperature for 30 minutes before counting (PBS I). The efficiency of maintained cooling was studied by keeping a parallel sample on ice for 30 minutes instead of incubation in room temperature (PBS II). Addition of 1 mM EDTA to maintained cooling suppressed engulfment to a baseline value reflecting a one-minute period of active phagocytosis (EDTA). Mean of 6 experiments, ±SEM

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Efficient Cell Mediated Lysis of Human Erythrocyte Target Cells Is Mediated by a Human Glycophorine A Specific Murine Monoclonal Antibody

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A human glycophorine A specific murine monoclonal antibody mediates the antibody dependent killing of antibody sensitized human erythrocytes by human K cells (monocyte-free mononuclear cells). The overall level of killing is lower than that mediated by conventional, human anti-D antibodies but it is significant on each target cell.

Keywords: monoclonal antibody, glycophorine A, ADCC

Introduction

Antibody dependent cellular cytotoxicity (ADCC) is a link between cellular and humoral immune mechanisms. Effector cells of non-T and non-B phenotypes – K cells – are armed with antigen specific antibodies and lyse the sensitized target cells.

It has been shown that human antibodies are very efficient in mediating this reaction, while mouse monoclonal reagents induced a negligible level of killing if any.

Here we describe the results obtained with a murine monoclonal antibody to glycophorine A, which mediates an efficient cell mediated lysis of sensitized human erythrocyte target cells.

Materials and Methods

A4 a murine monoclonal antibody used in the experiments, was obtained from a fusion described previously [1]. The antigens were the erythrocytes which contaminated the peripheral blood mononuclear cell suspension. Agglutinations were evaluated in normal (physiological saline) or low ionic strength solution (LISS) in the presence or absence of anti-mouse immunoglobulin [2].

The effector function of antibody in ADCC was tested on human erythrocytes as target cells and K cells as effector cells used at 1 : 5, 1 : 10 and 1 : 20 ratios, respectively. A4 monoclonal antibody was used at a final dilution of 1 : 40.

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The tests were performed in round-bottomed microtitre plates [3]. The specific ^{51}Cr release was calculated according to the following formula:

$$\text{specific } ^{51}\text{Cr release} = \frac{\text{test cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}} \times 100$$

Results and Discussion

A4 monoclonal antibody was selected on the basis of its capacity to induce strong agglutination of human glycophorine A positive erythrocytes. Glycophorine A negative erythrocytes were not agglutinated (personal communication of Patricia Tippett).

Monoclonal anti-glycophorine A antibody, A4, is of IgG1 isotype, and agglutinates papain treated erythrocytes at 37 °C in physiological saline and in LISS at room temperature, respectively, in the presence or absence of anti-mouse immunoglobulin.

Table 1

ADCC activity of A4 monoclonal antibody

Red cell type		LISS	LISS-AGT	ADCC test with A4			ADCC test with anti-D
				1 : 5	1 : 10	1 : 20	1 : 10
Rh(d)	1.	NT	NT	NT	1.2	NT	0
	2.	pos	pos	4.5	6.1	NT	0
Rh(D)	1.	pos	pos	0	0.7	1.7	65
	2.	pos	pos	1.3	1.8	2.3	33
	3.	pos	pos	5.0	8.0	6.9	30
	4.	pos	pos	3.1	6.8	7.0	56
	5.	pos	pos	6.4	8.0	8.1	61
	6.	pos	pos	5.1	7.0	6.9	37
	7.	pos	pos	3.3	7.4	5.1	25
	8.	pos	pos	5.2	8.6	7.6	56
	9.	pos	pos	4.6	9.6	8.8	73
	10.	pos	pos	5.1	8.0	6.9	62

NT = not tested

ADCC reaction was calculated at different target/effector cell ratios (1 : 5, 1 : 10 and 1 : 20) from triplicate culture samples.

1 : 40 dilution of cell culture supernatant

$$\text{ADCC reaction } (\%) = \frac{\text{test cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}} \times 100$$

The results are shown in Table 1. A4 monoclonal antibody mediates ADCC reaction on a broad variety of target cell types. The overall level of killing is, however, lower than that obtained by a human anti-D polyclonal typing reagent. This is presumable as polyclonal anti-D is able to bind multiple epitopes [4], although it is clear that A4 mediates killing.

It was shown previously in a mouse target cell system that monoclonal antibodies, reactive with various mouse cell surface antigens, mediate killing by human leukocyte effector cells [5]. The level of killing was variable depending on the specificity and the isotype of the antibody. Murine monoclonal anti-human antibodies of IgG3 isotype were found to be very effective in augmenting ADCC [6]. Human monoclonal anti-D antibodies of IgG1 isotype were effective in inducing lymphocyte (K cell)-mediated ADCC reaction [7].

We have broadened specificity by using a glycophorine A specific IgG1 murine monoclonal antibody in a human effector-target cell system. Generally, antibodies to the D antigen are used to evaluate the strength of ADCC reaction to K cell in clinical practice, however, the efficiency of killing depends on the Rh phenotype of the target cells.

A4 monoclonal antibody used in the described test system reacts with a narrow range of specificities, e.g. it is specific to the glycophorine A molecule on the human erythrocytes, which does not have a polymorphism similar to that of the Rh system. That is the reason why we have been able to get comparable levels of killing on a broad variety of erythrocyte target cells.

The system described here adds to the specificity of the ADCC reactions showing that the non-polymorphic human glycophorine A can also serve as a target antigen in K cell mediated killing.

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Serum Cholinesterase Variants in Spanish Blood Donors

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A population of 783 blood donors has been analyzed for serum cholinesterase variants resistant to dibucaine and fluoride. The incidence of these cholinesterase variants in the blood donors was lower than that found in other European countries.

Keywords: serum cholinesterase, genetic variants, Spanish blood donors

Introduction

The genetic polymorphism of serum cholinesterase (E.C.3.1.1.8) was first described by Bourne et al. (1952) and Forbat et al. (1953). The works of Kalow and Genest (1957), Harris and Whittaker (1961), and Liddell et al. (1962), have allowed the elaboration of a genetic model suitable for studying this agent. The biosynthesis of human serum cholinesterase (acetylcholine acetylhydrolase) is controlled by four allelic genes which function at the locus E_1^u. These are E_1^u for the usual type, E_1^a for the dibucaine-resistant type, E_1^f for the fluoride-resistant type and E_1^s for the silent gene. The C_5^+ variant of serum cholinesterase is controlled by the E_2 locus and can be identified only by electrophoretic techniques, Harris et al. (1963). It seems to be completely independent of all other variants.

The analysis of the genetic polymorphism of serum cholinesterase is important for two reasons: first, the frequency and distribution of the cholinesterase types in a population is interesting; and secondly, due to the various mutations of this enzyme, therapeutical accidents may occur (sometimes severe apnoea as a result of the administration of suxamethonium or derivatives of succinylcholine in general anesthesia), or toxic symptoms may arise (due to the use of chemical derivatives or anticholinesterase pesticides in industry or agriculture).

The object of this work was to determine the frequency of serum cholinesterase variants resistant to dibucaine (E^a) and to sodium fluoride (E^f), in a population of blood donors of the Instituto Español de Hematologia y Hemoterapia of Madrid.

Another aim was to contribute data which may reduce one of the many risks of blood transfusions.

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Materials and Methods

The 783 persons involved in this study were blood donors, 557 men and 226 women, 21 to 65 years of age.

Samples of 5 ml venous blood were collected in tubes without anticoagulant, and left to coagulate for 1 to 2 hours at 37 °C. Thereafter, they were centrifuged at 2.000 r.p.m. for 10 min., and the serum without visible hemoglobin was aspirated and transferred to sterile tubes. Sera with signs of hemolysis were not used in the assay. The evaluations were made on the same day as the collecting of the samples.

Serum cholinesterase activity was assayed by measuring the rate of hydrolysis of 5×10^{-5}M benzoylcholin chloride photometrically at 240 nm in a M/15 phosphate buffer pH 7.4 at 26.5 °C by the method of Kalow and Lindsay (1955). Dibucaine numbers, i. e. percentage inhibition of enzymic activity produced by 10^{-5}M dibucaine, were measured by the method of Kalow and Genest (1957) and fluoride numbers (percentage inhibition of enzymic activity produced by 5×10^{5}M sodium fluoride) according to Harris and Whittaker (1961). Both DN and FN are characteristic constants for each individual: the normal homozygotes (E_1^u, E_1^u) have a DN of 80 $\pm$ 3; in the normal and atypical enzymes of the heterozygotes, the DN is 62 $\pm$ 8; and in the atypical homozygotes the DN is 22 $\pm$ 6. The numbers corresponding to fluoride are, respectively: 61 $\pm$ 6; 48 $\pm$ 9; and 23 $\pm$ 5.

Results

Of the 783 individuals analysed in this work, 773 (98.72%) were the normal type $E_1^uE_1^u$; 7 (0.89%) were $E_1^uE_1^a$; and no individuals were found to be $E_1^aE_1^a$. The genetic frequency for E^a was 0.0044. There were 3(0.384%) individuals that were $E_1^uE_1^f$; and no individuals were type $E_1^fE_1^f$. The genetic frequency for E_1^f was 0.0019.

Table 1

Genotype	N° of individuals	DN (dibucaine number)	
		Mean	Standard deviation
$E_1^uE_1^u$	773	80.31	1.40
$E_1^uE_1^a$	7	64.50	0.41
$E_1^aE_1^a$	0	—	—

In Table 1 the means and standard deviation of the DN obtained in the distinct genotypes are given.

In Table 2 the means and standard deviation of the FN obtained in the distinct genotypes are shown.

Table 2

Genotype	N° of individuals	FN (fluoride number)	
		Mean	Standard deviation
$E_1^u E_1^u$	773	60.55	1.40
$E_1^u E_1^F$	3	52.00	0.87
$E_1^F E_1^F$	0	—	—

Discussion

Our average values for the DN and FN are in agreement with values reported by other authors in different ethnic groups.

Population genetic studies on cholinesterase polymorphism revealed an interesting general observation: all cholinesterase variants seem to be very rare in Negroid populations. The allele E_1^a (variant resistant to Dibucaine) is most frequent in Causasoids, whereas the frequency is relatively low in all the other human race groups. Information concerning the fluoride-resistant and the variant of the "silent" gene is rather limited. Although both of them are very rare, some authors found remarkably high frequencies in various populations (E_1^s in several Eskimo groups according to Scott et al. 1970 and Gutsche et al. 1967; E_1^f in Punjab population, according to Singh et al., 1971).

Table 3

Incidence of serum cholinesterase dibucaine-resistant variant in European populations

Population	N° Indiv.	Homozyg.	Heterozyg.	Gene Frequency of E_1^a	Author
French	1.522	—	57	0.0187	Schaap et al. (1967)
Yugoslavs	248	—	7	0.0141	Fraser et al. (1966)
Portuguese	179	—	6	0.0168	Kattamis et al. (1962)
Greeks	360	—	13	0.0181	Kattamis et al. (1962)
Greeks	561	—	16	0.0143	Morrow et al. (1965)
Greeks	218	1	10	0.0252	Neumann et al. (1968)
Bulgarians	108	—	2	0.0093	Steegmüller (1975)
Czechoslovakians	262	—	19	0.0362	Goedde et al. (1963)
Germans (Berlin)	1.000	—	23	0.0115	Prokop (1971)
Germans	280	—	7	0.0125	Steegmüller (1975)
British	703	—	27	0.0192	Kattamis et al. (1962)
Danish	1.278	1	32	0.0133	Hanel et al. (1978)
Spanish (donors)	783	—	7	0.0044	Present study

The distribution of the C_5^+ component does not show a clear geographical or racial variation. In many populations of different racial or geographical origin similar frequencies could be detected, yet with respect to average values, it can be stated that the highest incidence of C^{5+} is found among the Caucasoids.

The frequencies obtained in our donor population correspond to previous affirmations: the genetic frequency for the variant resistant to dibucaine is 0.0044, the genetic frequency of the variant resistant to fluoride is 0.0019.

Table 3 shows the incidence of the cholinesterase resistant to dibucaine in the different European populations. In our donor population its frequency is the lowest. Table 4 shows the incidence of fluoride-resistant cholinesterase in some

Table 4

Incidence of serum cholinesterase fluoride-resistant variant in European populations

Population	N° Indiv.	Homozyg.	Heterozyg.	Frequency of gene E_1^f	Author
Germans	801	—	12	0.0075	Goedde et al. (1964)
Germans	280	—	4	0.0071	Steegmüller (1975)
Bulgarians	108	—	1	0.0046	Steegmüller (1975)
Icelanders	128	—	3	0.0117	Neumann et al. (1968)
Greeks	218	—	7	0.0161	Neumann et al. (1968)
Danish	1.278	—	3	0.0012	Hanel et al. (1978)
British students	780	—	5	0.0032	Whittaker (1968)
Spanish (donors)	783	—	3	0.0019	Present study.

European populations; the frequency in our studied population was low, only the Danish population showed a lower incidence. Based on these results and values obtained in 1973 by Goedde et al., 1973 for Andalucia (0.0040 for E_1^a) and Spain Central Meseta population (0.0094) we suggest that these low frequencies are due to a racial or anthropological characteristic of the Spanish population.

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Adhesive Properties of Blood Cells

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The adhesive interactions of the cells with each other and with the extracellular matrix (ECM) are important for their normal growth and differentiation. Currently, very little is known concerning the adhesive and cell recognition factors that maintain cell-to-cell contacts in haemopoietic tissues. The majority of peripherial blood cells are known to possess an ability to interact with each other (aggregation phenomenon) or with various artificial materials, which has important implications for medicine, particularly in the fields of thrombosis, vascular prosthetics and dialysis. On the other hand, recent studies indicate that blood cells can interact with the main important components of ECM, such as collagen, fibronectin, etc. These data, as well as the newly discovered receptor structures for various adhesive proteins on the surface of blood cells suggest that the haemopoietic tissue has a diverse function involving interaction with the ECM.

Keywords: adhesive protein receptors, cell attachment proteins, cell-to-cell and cell-to-matrix interactions, haemopoietic tissues, lymphoid and myeloid cells

Introduction

The haemopoietic tissues are situated in different parts of the body. All mature and some immature myeloid and lymphoid cells circulate because they exist in a free and mobile state.

However, in some stages of haemopoiesis they require a certain immobility as does every tissue cell. This is provided by specific cell-to-cell and cell-to-matrix interactions [1]. The adhesive cell-to-cell contacts take part in the processes of leukocyte migration into extravascular tissues, T-lymphocyte interaction with target cells, homing and recirculation of lymphoid cells [1, 2]. The interactions between cell-to-matrix adhesive receptors and their cognate protein ligands are essential for the movement of haemopoietic cells from and to the bone marrow and lymphoid tissues during the process of their differentiation and maturation. They are important also for the mobility of some mature blood cells during their penetration and functioning in the extravascular space [2].

In this brief review we summarize the recent data in this field and try to clarify the relationship between the adhesive properties of blood cells and some of their functions.

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General Adhesive Receptors on the Surface of Blood Cells and Their Cognate Ligands

It has become apparent that several specialized cell membrane structures, similarly to the antigen-binding receptors on lymphoid cells, may serve as recognition sites and convey crucial signals to haemopoietic cells upon appropriate occupation by ligands. The mechanism by which haemopoietic cells recognize and adhere to specific sites in the ECM, as well as to other cells, has been intensively investigated during the past few years [2, 3]. There is evidence, indicating that several cell surface receptors from the integrin superfamily of cell adhesion molecules (I-SCAM) are located on the surface of blood cells [2]. The I-ESCAM proteins include cell-to-matrix and cell-to-cell adhesion receptors-heterodimers

Table 1

"Integrin superfamily" of adhesive protein receptors

Receptor	Cognate ligand	Distribution	Function
VLA-1	?	T*-ly, liver, muscle skin, fibroblasts, HTLV-IT, NHCL	not normal(?) arthrit. patients
VLA-2	collagen	T*-ly, platelets	coagulation
VLA-3	collagen, laminin Fn	kidney, thyroid Ly, Mo, basement membranes, preMo	T-cell ontogeny, malignant transformation, haemopoietic cells, metastasis
VLA-4	?	thymus, bone marrow, most HCL, (T, B, myelomas)	anchorage of lymphocytes in bone marrow
VLA-5	Fn	Ly, Mo, platelets, thymus, bone marrow	blood cell maturation, homing phenomena, anchorage of blood cells
VLA-6	?	mammary epithelial cells	unknown (? cell-ECM interaction)
GpIIb/IIIa	vitronectin, Fn, Fng, VWF	platelets	coagulation
LFA-1	GAP	Ly	antigen-independent Ly, adhesion, immunological response
Mac-1	?C3bi	Mo, granulocytes	adhesion to endothelial cells in vessel wall
p150, 95	?C3bi	Mo, granulocytes	adhesion to endothelial cells in vessel wall

Ly: lymphocytes, *: activated Ly, Fng: fibrinogen, GAP: general adhesive protein, Mo: monocytes, HTLV-IT and NHCL: lymphoid cell lines, other abbreviations: see in text

conditionally divided into three subgroups (Table 1): (i) VLA (Very late antigens) [2], (ii) lymphocyte functional antigen-1 (LFA-1); macrophage antigen-1 (Mac-1); and p-150,95 proteins [4]; (iii) major surface glycoproteins [5]. All or most of these receptors can recognize ligands which contain Arg-Gly-Asp or closely related amino acid sequences [1, 2]. It is extremely interesting that the I-ESCAM proteins exhibit a significant structural homology, and are also homologous to other proteins, such as the immunoglobulin superfamily, major histocompatibility antigens, some hormonal receptors, as well as most cell attachment proteins [32]. This suggests a close genetical relation between them, which, however, is beyond the frame of this review. According to Edelman [32] most of these proteins originate from the exone conversion (or duplication) of the more ancient I-ESCAM superfamily gene.

Which are the known adhesive ligands in the haemopoietic tissues?

According to the latest data, the bone marrow stroma consists of various attachment proteins, such as type I, III, and IV collagens, fibronectin (Fn), laminin, and proteoglycans [6]. These proteins are also structural components of the vessel wall [7, 8]. In soluble form Fn, and vitronectin are major cell-adhesive glycoproteins, present in mammalian plasma [7, 8].

Up to now, more than 10 types of collagen have been well characterized. Collagen types I and III are the major collagenous components of the vessel wall [8], while collagen types IV and V are predominantly restricted to the basement membranes. Immunohistochemical studies suggest a close association of collagen types I and III to Fn, while a second structural adhesive glycoprotein-laminin is colocalized with collagen types IV and V [7, 8].

Perhaps the most studied component of the ECM is Fn [7]. This glycoprotein dimer (mol.w. 220 kDa) is predominantly associated with the ECM of many tissues, and can act as a bifunctional ligand between cells and collagen [7]. It is widely accepted that Fn participates in numerous biological phenomena, including cell adhesion and migration, embryonic differentiation, malignant transformation, wound healing, etc. [7].

The basement membranes are also adhesive extracellular matrices, and consist mainly of collagenous and noncollagenous proteins. The latter are still incompletely characterized, but are known to include a great amount of laminin [9]. It consists of three polypeptide chains (A, B1, B2) linked by disulfide bonds, which migrate on SDS polyacrylamide gels under reducing conditions with apparent molecular weights of 400 kDa, and 210, 230 kDa, respectively.

The other adhesive protein, vitronectin, is a monomeric glycoprotein detected as a mixture of 75 kDa and 65 kDa polypeptides [10]. Vitronectin binds to heparin, collagen, and a variety of cultured cells. It mediates biological effects in vitro similar to those of Fn and laminin [10].

Among the adhesive protein receptors on blood cells, the VLA-1 and VLA-2 were originally defined by Hemler et al. in 1983 as heterodimers appearing 2–4 weeks after in vitro activation of T lymphocytes [11]. Subsequently three additional heterodimers (VLA-3, VLA-4 and VLA-5) were discovered,

and found to belong to the same protein family [12]. A sixth distinct heterodimer VLA-6 has recently been discovered, but its influence on haemopoietic cells has not yet been analysed [13]. The results of biochemical and immunological studies indicate that each VLA protein is composed of distinct α-subunit, which is noncovalently associated with a common β-subunit [12]. Amino-terminal sequence analysis of the five VLA β-subunits revealed striking homologies within the group as well as with β-subunits from heterodimers belonging to the other families [2].

Except for granulocytes and red (mature) blood cells, nearly all cell types express one or more VLA complexes (Table 1). VLA-1 and VLA-2 expression is restricted to activated T lymphocytes and a few T lymphoblastoid cell lines [11], whereas VLA-4 is expressed on nearly all lymphocytes, monocytes and related cell lines [12]. Both VLA-2 and VLA-5 are measured on activated platelets [13]. VLA-5 is present on the myeloid cell lines K-562 and K-937, and is immunochemically identical with the Fn receptor [14]. A combination of functional and structural approaches suggests that similar Fn receptors are present in many haemopoietic cells, for example bone marrow cells [6] and several pre B-lymphoid cells and B-myelomas [15] immature thymocytes [16], T-lymphoma [17], monocytes and macrophages [18], proerythroid cells and erythroid cell lines [22]. The data obtained in our laboratory also showed that a subpopulation of human peripherial lymphocytes [19] and rat lymphocytes from different lymphoid tissues [20] possess a similar Fn-binding capacity.

LFA-1, Mac-1 and p-150,95 adhesive receptors of the second integrin family subgroup, are also composed of noncovalently linked heterodimers, which also have distinct α-subunits with mol.w. of 180, 170 and 150 kDa, respectively [2, 4]. The β-subunit (mol.w. 95 kDa) has been shown to be identical in all three proteins. LFA-1 is typical for lymphocytes, while Mac-1 and p-150,95 are expressed on the surface of monocytes and granulocytes [2].

Various cytoadhesive proteins have been discovered on platelet membranes during the past few years [5]. According to the "classical concept" the platelets possess numerous glycoproteins (Gp) divided into three large groups – Gp I, II and III [5]. In general, Gp Ib is responsible for the platelet adhesion to the endothelial cover of the vessel wall [13], while Gp IIb and III are related to platelet aggregation phenomena [5]. Gp IIb and IIIa, exist as a noncovalent complex in the platelet membrane, and are one of the most studied members of I-ESCAM (Table 1). This glycoprotein complex serves as a receptor for four proteins: Fn, fibrinogen, Von Willebrand factor, and vitronectin [1, 2].

Other specific adhesive receptors besides the integrin family members are also present on the surface of lymphocytes [21]. These receptors are related to the capacity of these cells to locate and enter lymphoid organs by a series of adhesive interactions called "homing" phenomena [21]. The lymphocyte homing receptors are cell surface glycoproteins. They are responsible for the adherence to cognate ligands, located on the high endothelial vessels (HEV-ligands). The mouse lymph-node homing receptor is an extensively glycosylated branched-chain polypeptide [21].

Adhesive Interaction and Haemopoiesis

Blood cell production is a developmental process requiring close cell-to-cell association between haemopoietic cells and their supportive stromal cells. These stromal cells secrete regulatory factors that promote differentiation and maturation of blood cells. Currently, very little is known about the adhesive and cell recognition factors maintaining cell-to-cell contacts in haemopoietic tissues. Since actively dividing haemopoietic progenitor cells are preferentially associated with the stromal layer of bone marrow cultures [23], it is possible that certain components of this matrix are important for the maintenance of haemopoiesis. This possibility is suggested by the observation that inhibitors of the matrix formation decrease stem cell production, whereas compounds that stimulate matrix production increase stem cell proliferation [23].

The most studied component of ECM, associated with haemopoiesis, is Fn [7]. The immunofluorescence studies of the developing bone have indicated that haemopoietic colonies are associated with Fn-rich areas of the bone marrow [6, 23]. This suggests a possible important role of this protein in anchoring haemopoietic cells to the stroma. Bone marrow precursors of erythrocytes interact with Fn and the loss of Fn adhesion results in the release of the cells into the circulation [22]. The data showing that both immature erythroid and myeloid cell lines can adhere to Fn [23] are in agreement with the above concept. Adhesion occurs via the same cell attachment domain (110 kDa fragment) that functions in the fibroblast adhesion [23]. Moreover antibodies to the 145 kDa plasma membrane glycoprotein (structurally and immunologically similar to fibroblast Fn receptor) can prevent haemopoietic cell adhesion [23]. It was shown that human, rabbit and mouse reticulocytes attach specifically to Fn [22]. Remodelling of the reticulocyte plasma membrane during maturation in vivo is indeed accompanied by the loss of adhesion to Fn [22]. The spleen is one of the sites where reticulocytes lose their ability to attach to Fn [22].

The adhesion is also an essential mechanism for T lymphocyte differentiation in the lymphoid organs [21]. Recent data [6] show that the Fn receptor structures are preferentially expressed in the proliferating subpopulations of thymocytes. Based on these data, it appears that the Fn receptor activity is important during the early phase of thymocyte development, and disappears after cell differentiation [16, 17].

There are insufficient data concerning the role of other matrix proteins. In general these studies reported a weak adhesion of haemopoietic cells to laminin and collagen [6], but further investigations are necessary in this field.

Adhesive Properties of Lymphocytes

Although lymphocytes are relatively nonadhesive and immotile in circulation or in vitro conditions, they acquire these characteristics after penetration

into the tissues [3]. The lymphocytes undergo adhesion to endothelial cells and migration is observed in vivo under certain circumstances [21]. The factors responsible for these changes are however unknown. Some adhesive receptors binding the lymphocytes, and molecules such as Fn in the ECM regulate the adhesion and locomotion of the contacting cells. It seems that Fn is the most important protein involved in the lymphocyte adhesive interactions. Gardarelly and Pierschbacher [16] were among the first authors who discussed the role of Fn in lymphocyte adhesion. As mentioned above, the results obtained in our laboratory suggest the presence of a subpopulation of human peripherial lymphocytes, which shows an ability to interact with both immobilized and soluble Fn [19]. In other experiments with rats [20] we demonstrated the important role of Fn in the lymphocytes anchorage during their functional distribution in the peripheral lymphoid tissues. An investigation of 12 lymphoid cell lines showed that 5 of them, adhering to Fn, had B-cell characteristics, while neither T-cell lines nor pre B-cell lines were observed to adhere [15]. Kurki et al. [24], however, reported that T-lymphocytes adhere predominantly to Fn, and the mitogen stimulation additionally promotes this phenomenon.

In a previous paper [19] we reported on the inhibition of lymphocyte adhesion on Fn-coated surface in the presence of soluble Fn. We suppose that interaction of the lymphocyte Fn receptor with solid phase-immobilized Fn leads to cell adhesion, while preliminary saturation of the receptor inhibits the process. As shown in Table 1, there are at least three receptors for Fn on the surface of haemopoietic cells (VLA-3, -5 and Gp IIb/IIIa), but with the exception VLA-5, their presence on lymphocytes has not been studied [1].

We found no information concerning the adhesive interaction of lymphocytes with other matrix components, such as laminin, vitronectin or proteoglycans. It seems that lymphocytes adhere poorly to collagen, but they are able to attach to and migrate in three dimensional collagen matrices [1, 3].

As metioned above, the lymphocytes have various adhesive receptors which mediate cell-to-cell interactions that are important for the realisation of homing phenomena [21]. At present, at least two independent homing receptors exist in vertebrates lymphocytes: one for peripherial lymphnode vessels, and another for Payer patch vessels (or appendix in humans) [21]. Other authors [21] demonstrated that lymphocytes adhere to the endothelial cells in vitro as a result of specific receptor-ligand interactions. According to the latest data LFA-1 is such a receptor (see above). After the interaction the cells transmigrate across the endothelial cell layer into the lymphoid organ tissue [8], B cells moving vectorially toward the follicules (B cell domain), and T-cells toward the T-cell domains [21].

The specific movement of lymphocytes from blood to tissues occurs not only during their normal traffic of recirculation and homing, but also when the effector T lymphocytes enter sites of inflammation, for example virus infection or allografts [21]. In chronic inflammatory sites on the blood vessels endothelial cells there are receptors similar in structure to the normal lymphocyte homing receptor [27].

Macrophage, Monocyte, and Neutrophil Adhesion

It seems that the macrophages (monocytes), as well as the neutrophils display both active and passive modes of adhesion. The active response of macrophages to the substrate is probably a manifestation of their phagocytic properties. It has been suggested, that the spreading of macrophages on a substrate is an attempt of the cells to phagocytose a particle of infinite diameter [3].

Adhesion to the substrate, as well as the spreading reaction, has been shown to require serum in experiments with both neutrophils and monocytes [3]. Macrophages can normally synthesize Fn [18]. This protein is a normal component of the surface coat of human neutrophils [3]. Binding of Fn to the neutrophils (via a specific receptor – see above), results in the activation of the phagocytic response of these cells, and induces the production of a chemotactic factor, stimulating neutrophil substrate adhesion [25]. Fn has also been shown to promote the attachment of Staphylococcus aureus to neutrophils, even though it was not able to promote neutrophil phagocytosis of bacteria [3].

Little information is available on the interaction of macrophages and neutrophils with other components of the ECM. Immunocytochemical attempts to identify laminin were unsuccessful [6]. Nevertheless, monocytes and macrophages can interact with collagen [26], and this results in an augmented secretion of cytokines and prostaglandins. Evidence for a monocyte (macrophage) collagen receptor was obtained through affinity isolation of a 125/150/180 kDa structure from the promonocyte cell line U-937 [1].

The specific I-ESCAM receptors (Mac-1 and P150,95) on the surface of macrophages and granulocytes are responsible for their interaction with the endothelial cells. Mac-1 is also a receptor for the C3bi component of complement and is simultaneously involved in monocyte and neutrophil adhesion. The second antigen, P150,95 appears to have functions similar to those of Mac-1, but displays somewhat different cell distribution [1, 2]. The importance of the Mac-1/p150,95 family in leukocyte function is underestimated due to the existence of the human genetic disease termed leukocyte adhesion deficiency (LAD) [27]. Patients with LAD are deficient in their cell surface expression of LFA-1, Mac-1 and p150,95 [2] and suffer from recurrent life-threatening bacterial infections. Leukocytes from these patients show profound defects in virtually all adhesion-related functions [27].

Platelet Adhesion

The adhesion of platelets to a substrate is the initiating event in their basic function that results in the release of platelet content and platelet aggregation, which in turn leads to clot formation [3]. Because of the importance of platelet adhesive interactions in vivo, sophisticated strategies for studying adhesion and aggregation under in vitro conditions have been developed. In this paper we discuss platelet substrate adhesion only.

The identity of the connective tissue components that are the substrates for platelet adhesion is controversial [3]. Following trauma, platelets adhere to the underlying connective tissues, especially to those containing collagen [28]. The interaction of platelets with collagen is an important early event in haemostasis and thrombosis. According to the "classical concept" the adhesion of platelets to collagen exposed in injured blood vessels is the first step of a complex process leading to platelet activation and clot formation. Native collagen is a substrate for platelet adhesion, whereas thermally denaturated collagen is not [28]. It seems that most probable candidate for a specific collagen receptor structure on the platelet surface is VLA-2 (see above), possibly located in a Gp Ia–IIa fraction [13].

Fn is also the substrate for platelet adhesion and can participate in the mechanisms of platelet aggregation with collagen [29]. Platelet α-granules also contain a significant amount of Fn which can be released during aggregation. This activity is realised by two separate receptors: VLA-5, which is specific for Fn, and Gp IIb–IIIa, which shares Fn binding activity with three other adhesive proteins (vitronectin, fibrinogen and Von Willebrand factor [2, 12]. The possible role of adsorbed fibrinogen in platelet adhesion has been mentioned previously [30].

Another plasma protein necessary for platelet adhesion is the Von Willebrand's factor [31]. Patients lacking this protein exhibit inefficient platelet-substrate adhesion [2, 31]. The functional importance of membrane Gp IIb–IIIa on the surface of human platelets is indicated by their lack or severe decrease of human platelets from patients with Glanzmann's thrombasthenia [31]. These platelets are unable to aggregate in response to reagents such as ADP, thrombin, collagen or epinephrine.

Platelet-subendothelial adhesion is impaired also in patients with Bernard–Soulier syndrome [2]. In this case certain membrane glycoproteins present in normal platelets, are apparently missing.

In conclusion blood cells seem to possess a greater variety of cell-to-matrix and cell-to-cell adhesion capabilities than suspected previously. It should be emphasized that the above interactions are quite likely to play an important role in the functional behaviour of these cells. In our opinion future investigations should be aimed at: (i): studying the adhesive receptor phenotype of blood cells, especially that of lymphocytes; (ii): demonstrating cell-environmental adhesion relations in the processes of myelopoiesis and lymphopoiesis. Moreover, it would be of great importance to elucidate the role of the adhesive events in both normal haemopoiesis and processes of malignant transformation and dissemination in neoplastic haemopathies; (iii): clarifying the role of the adhesive properties of immature and mature blood cells in their circulation, homing and functioning.

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